Studies on the carboxyl-terminal amino acid sequence of the chaperonin GroEL from *Escherichia coli*.

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To my Mother and late Father

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Abstract

Previous work from our laboratory had suggested that the carboxyl-terminus of GroEL limited the ability of this protein to reverse the temperature-sensitive defects of *dnaA*^{ts} mutations in *E. coli*. This carboxyl-terminus consists of a 13 amino acid string of glycine and methionine residues, a motif that is highly conserved amongst many of the GroEL homologues sequenced so far. A clone was constructed which expressed a form of GroEL lacking this motif. Surprisingly this clone (which also expressed wild-type *groES*) was unable to suppress *dnaA*^{ts} mutations when overexpressed, but was able to complement *groEL*^{ts} mutations (even when present in single copy). A *groE* deletion mutant strain was constructed and this too was complemented by the truncated form of *groEL*. The resulting strain, carrying only truncated *groEL*, was extensively characterized and found to behave identically to an isogenic strain carrying wild-type *groEL*, in that growth rates, temperature dependence, carbon source utilization, bacteriophage sensitivity, ethanol sensitivity, UV sensitivity and β-lactamase excretion were all found to be identical between the two strains. However, competition experiments involving co-culturing of the strains carrying *groEL*$^{+}$ and *groEL*$_{\mathbf{tr}}$ demonstrated an advantage to the cells expressing the wild-type gene when grown at 42°C. The advantage was found to be due to the strain encoding the truncated *groEL* exiting stationary phase and entering log-phase growth more slowly than cells expressing *groEL*$^{+}$. *In vitro* analysis of truncated GroEL showed that its ATPase activity was slightly reduced when compared to the wild-type protein, and this observation can be used to account for the differences seen *in vivo*. It was also found that strains expressing *groEL*$_{\mathbf{tr}}$ are more sensitive to the dye crystal violet, and low levels of some other biocides at 42°C. This suggests a role for the GroE proteins in membrane biogenesis and/or maintenance as well as an involvement of the glycine–methionine sequence.

Further truncations of the *groEL* gene were made and the expressed proteins tested for *in vivo* activity. A truncation lacking 31 carboxyl-terminus codons (*groEL517*) did not complement *groEL* mutations; however, the truncated subunits produced from this gene were assembly competent and found associated with wild-type GroEL protein in the tetradecamers characteristic of the active form of GroEL. A second truncation lacking 43 amino acids (*groEL505*) produced an insoluble polypeptide not associated with assembled GroEL particles. This suggests that the amino acids between 505–517 are essential for folding of GroEL monomers, or their assembly into GroEL particles.

Abbreviations

ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
Amp	Ampicillin
ATP	Adenosine 5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolylphosphate
bp	Base pair
BSA	Bovine serum albumin
cAMP	Cyclic AMP
Chl	Chloramphenicol
CsCl	Cesium chloride
CTP	Cytosine 5'-triphosphate
dATP	Deoxy ATP
ddATP	Dideoxy ATP
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Diaminoethanetetraacetic acid
EtBr	Ethidium bromide
GTP	Guanosine 5'-triphosphate
IPTG	isopropyl-β-D-thiogalactoside
Kan	Kanamycin
kb	Kilobase
kDa	Kilodalton
mRNA	Messanger RNA
OD_w	Optical density $_{\text{wavelength}}$
PAGE	Polyacrylamide gel electrophoresis
PIPES	Piperazine N,N'bis (2-ethane sulphonic acid)
PMSF	Phenylmethylsulphonyl fluoride
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
Spc	Spectinomycin
ssDNA	Single-stranded DNA
Str	Streptomycin

TEMED	N,N,N',N',-tetramethylethylethylenediamine
Tet	Tetracyclin
Tris	Tris(hydroxymethyl)aminomethane
Tmp	Trimethoprim
ts	Temperature sensitive
TTP	Thymidine 5'-triphosphate
UTP	Uridine 5'-triphosphate
v:v	Volume by volume
w:v	Weight by volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
X^R	Resistance to antibiotic X
X^S	Sensitive to antibiotic X

Chapter 1
Introduction

1.1 The *Escherichia coli* heat-shock response

The normal temperature range for the Gram-negative bacterium *Escherichia coli* has been regarded as being between 20 and 37°C. Between these temperatures, growth rates are a simple function of temperature and fluctuations of temperature within this range are instantaneously catered for by minor changes in cell physiology. Below 20°C the growth rates are normally reduced and shifting cells from the normal range to these low-growth-rate temperatures results in a lag in growth before the final rate is attained. Shifting cells beyond the upper limit of the normal range does not show such a growth lag but analysis of such cells has shown major changes in their cellular composition (Neidhardt and VanBogelen, 1987).

Shifting growing cells from normal temperatures to above 40°C results in the synthesis of large quantities of certain proteins within 1–2 minutes. For most other proteins synthesis is transiently halted. This response quickly ceases if the cells are returned to the normal temperature range, and even if the cells are maintained at the higher temperature the rate of production of the induced proteins diminishes although a new steady-state level higher than that at 37°C is usually maintained. The proteins which are induced in this way are termed 'heat-shock proteins' (HSPs) (Table 1.1.1). The heat-shock response was first described when a specific puffing-pattern was observed on polytene *Drosophila* chromosomes upon heat treatment (Ritossa, 1962). The response is now known to occur in animals, plants and eukaryotic microbes, and its discovery in prokaryotic organisms led to the realization of its ubiquity. In fact, not only is the response virtually universal, but many of its components are as well. Bacterial HSPs show strong sequence homology at both DNA and protein levels to proteins found in higher eukaryotic organisms (for an example see Waldinger *et al.*, 1988). This suggests that HSPs could have important fundamental roles within all cell types. (See Lindquist and Craig (1988) and Craig *et al.* (1993) for HSP reviews.) As ever, *E. coli* has been a major tool for elucidating what is currently known about the biology of the heat-shock response and its protein components. The response is also an excellent model for studying control and regulation of gene expression.

Table 1.1.1 Heat-shock proteins of *E. coli*.

Gene	Protein name(s)	Mol wt[a]	Abundance[b] (α', 10^3)	Induction ratio[c]
grpE	GrpE	25,300	1.44	9.5
groEL (*mopA*)	GroEL	57,300	16.47	7.9
groES (*mopB*)	GroES	10,400	2.61	19
dnaK	DnaK	69,100	14.09	13
dnaJ	DnaJ	41,000	<0.2	ND
rpoD	Sigma-70	70,300	2-3	ND
htpE		14,700	0.87	74
htpG		71,000	2.61	26
htpH		33,400	1-2	11
htpI		48,500	1-2	ND
lysU	Lysyl-tRNA synthetase II	60,500	0.18	10
htpK		10,100	<0.1	45
htpL		21,500	<0.2	6.4
htpM		84,100	<0.73	10
htpN		13,500	<0.2	56
htpO		21,000	<0.1	25
lon	Lon, La	94,000	1.61	12

a. Apparent molecular weight from either analysis of polyacrylamide gels, or sequence data where available.
b. Expressed as a', the weight fraction of each protein relative to total protein, in glucose-rich medium, 37°C.
c. Determined by dividing the amount of [^{35}S]-methionine incorporated into a given polypeptide spot from 3–8 min after a shift from 28–42°C by the amount incorporated in a 5-min period before the shift. All values were corrected for differences in cell density. ND, not determined.
Table was adapted from Neidhardt and VanBogelen (1987), which should also be consulted for references. Note that very recently Chuang and Blattner (1993) have used Kohara phages to demonstrate the presence of at least 21 further heat-shock gene products not described in this table.

It is notable that the HSPs can be induced not only by heat, but also by other agents (Table 1.1.2). Interestingly, not all the heat-inducible HSPs are induced by all these agents, and each agent elicits the production of a characteristic spectrum of HSPs. All these causative agents place the cell under considerable stress and it seems that cellular stress is the common factor for production of HSPs. It seems reasonable, therefore, to suppose that HSPs are produced to protect the cell from environmental stress. However, evidence is accumulating that this view of HSP function is far too simplistic, and

some of the HSPs are required for cellular viability at all growth temperatures.

Table 1.1.2 Agents that induce the heat-shock response in *E. coli*.

Inducing agents[a]	**Effects on cell structures or function**
Shift from 28 to 42°C	Growth rate doubling; transient inhibition of cell division; general alteration of gene expression; increase in ppGpp accumulation.
Shift from 28 to 50°C	Single- and double-stranded breaks in DNA; inactivation of many enzymes and macromolecular synthesis; damage to cytoplasmic membrane; degradation of RNA, DNA and proteins.
Ethanol	Mistranslation; disruption of membrane transport and translocation; increase in ppGpp accumulation.
Puromycin	Premature chain termination and release of peptidyl puromycin from ribosomes.
Viral infection	Inhibition of host RNA, DNA, and protein synthesis; decrease in accumulation of cAMP; increase in ppGpp accumulation.
Nalidixic acid	Inhibition of DNA gyrase activity, thus blocking replication.
Methylating and alkylating agents	Inactivation through modification of nucleic acids and proteins in general.
Cadmium chloride	Single-stranded breaks in DNA; inactivation of proteins.
Hydrogen peroxide	Depletion of cellular glutathione; direct damage to DNA; general inactivation of proteins.
Amino acid restriction	Increased accumulation of ppGpp and decreased synthesis of stable RNA and protein.

a. The agents are listed roughly in order of effectiveness as inducers from strong to weak.
Table adapted from Neidhardt and VanBogelen (1987), which should also be consulted for references.

1.2 Regulation of the heat-shock response in *E. coli*

The heat-shock response in *E. coli* was first discovered genetically in a mutant carrying a temperature-sensitive nonsense suppressor (Cooper and Ruettinger, 1975). Analysis of this mutant showed that it was unable to produce a number of proteins when shifted to non-permissive temperatures. The mutation could be complemented by a plasmid carrying the 76 minute region of the *E. coli* chromosome resulting in renewed expression of the HTP (high temperature production) proteins (Yamamori and Yura, 1980; Neidhardt and VanBogelen, 1981). The gene responsible was characterized as a positive regulator of the HTP proteins and was called *htpR* (Yamamori *et al.*, 1982; Neidhardt *et al.*, 1983). It is now more commonly known as *rpoH* and codes for the RNA polymerase subunit sigma-32 (σ-32). The HTP proteins mentioned above are, of course, the heat-shock proteins and expression of the regulon requires σ-32-containing RNA polymerase. Analysis of DNA sequence upstream of HSP genes has uncovered a σ-32 recognition sequence distinct from the normal σ-70 –10 and –35 sequences (Cowing *et al.*, 1985). The carboxyl-terminus of σ-32 shares considerable sequence similarities with that of the common transcription factor σ-70 including the putative DNA contact domains (Landick *et al.*, 1984). Sigma-32 stimulates transcription of the HSPs in cells undergoing heat shock by displacing σ-70 from RNA polymerase.

Intracellular levels of σ-32 increase rapidly upon heat shock and promoter selectivity is therefore thought to be caused, at least in part, by increased σ-32 availability. *RpoH* mRNA levels increase five-fold upon heat-shock, although the rate of synthesis of the protein is only doubled (Grossman *et al.*, 1987). The half-life of the protein is greatly increased however, and levels of σ-32 can be as much as eight times that prior to induction; it is thus thought that some sort of post-transcriptional/translational control is occurring (Erickson *et al.*, 1987). The *rpoH* gene has a rather complex promoter structure with at least four transcriptional start sites. Three of these are recognized by σ-70-containing RNA polymerase and the other is transcribed by an ultra-high-temperature sigma factor, σ-E (Erickson and Gross, 1989; Wang and Kaguni, 1989b). At normal temperatures the σ-70

promoters are the most active, whereas at the potentially lethal temperature of 50°C all *rpoH* transcripts originate from the σ-E promoter. At this temperature σ-32 is unstable, but it has been postulated that *rpoH* mRNA availability at such temperatures could enhance cell viability by production of HSPs immediately upon temperature decrease.

Recently it has been shown that certain of the HSPs themselves are involved in regulating the heat-shock response by interacting directly with σ-32. It had been known that certain *dnaK* mutants of *E. coli* show an inability to switch off the heat-shock response once initiated, but the possible role of the DnaK protein either as an active or passive regulator was unknown (Tilly *et al.*, 1983). Gamer *et al.* (1992) have now shown that the HSPs DnaK, DnaJ and GrpE can bind to σ-32. DnaK/GrpE–σ-32 complexes can be dissociated upon ATP hydrolysis, but the DnaJ–σ-32 complex is ATP independent. A simple model is that these proteins bind σ-32 *in vivo* in the absence of cellular stress and render it a substrate for proteolytic degradation, but that in the stressed cell the HSPs are acting elsewhere and thus the σ-32 is free to interact with the RNA polymerase core and transcribe the heat-shock genes. Once the cellular damage subsides and the cellular environment returns to normal the HSPs in question can return to their role in binding and destabilizing σ-32. The actual interactions seen between these proteins are rather complicated and such a simple model does not fully explain the apparent complexity of the system. Nevertheless, what is obvious is that a negative-feedback mechanism exists which regulates σ-32, and therefore the heat-shock response.

Expression of the *rpoH* gene is also regulated by the DnaA protein. DnaA protein's apparent main function is the initiation of chromosomal DNA replication from *oriC*, but it is also thought to be involved with transcriptional repression of genes containing DnaA-binding sites (DnaA boxes) within promoter regions. *RpoH* has two such boxes and filter-binding studies together with DNase I footprinting have shown that these putative boxes do seem to bind DnaA protein (Wang and Kaguni, 1989a). Elevating the intracellular levels of DnaA causes repression of *rpoH* transcription from two of its promoters both *in vivo* and *in vitro*. DnaA possibly regulates its own

expression via DnaA boxes found within its own promoter (Wang and Kaguni, 1987), but it seems that the *rpoH* DnaA boxes have a higher affinity for DnaA than the boxes within the *dnaA* gene itself. So, DnaA protein can modulate σ-32 levels to some extent. This could be significant since some of the HSPs have been shown to be involved with DNA replication (Wada and Itikawa, 1984; Sakakibara, 1988; Bukau and Walker, 1989b; Hupp and Kaguni, 1993a, b).

It has been shown that in addition to temperature increase, alterations in DNA supercoiling at constant temperature influence *rpoH* expression from a plasmid template (Ueshima *et al.*, 1989). This is interesting since it has been suggested previously that DNA relaxation could be a stimulus for heat-shock-response induction (Travers and Mace, 1982). Indeed many of the agents that induce the HSPs are known to damage DNA (e.g. UV light, cadmium chloride) or interfere with DNA topology (e.g. gyrase and topoisomerase inhibitors). This DNA damage response is *recA* independent and *rpoH* dependent (Kruger and Walker, 1984). It has been shown that the genes involved with osmoregulation (*osm*) and the structural gene for DNA ligase in *E. coli* are both influenced by alterations in DNA supercoiling (Higgins *et al.*, 1988; Liebart *et al.*, 1989). It is therefore possible that *rpoH* could be regulated at the transcriptional level by local DNA topology. However, not all HSP-inducing agents are known to damage DNA directly, and so maybe this is only one of several 'primary' stimuli.

It has been found that abnormal polypeptide production by incorporation of canavanine (an arginine analogue), or by sensitivity to puromycin and streptomycin (which cause premature polypeptide chain termination and mistranslations, respectively), can induce some HSPs in an *rpoH*-dependent manner (Goff and Goldberg, 1985). The product of the heat-shock gene *lon*, the La protease, is possibly thought to play an important protective role against accumulation of aberrant proteins, which could be potentially harmful, or at least useless. Transcription of *lon* can be induced by the production of a single species of unstable polypeptide in *E. coli* (Goff and Goldberg, 1985). It is also believed that other HSPs are involved in protein degradation since many HSP mutants have slower rates of proteolysis than wild-type controls (Straus *et al.*, 1988).

Despite much research, a unifying primary inducer of *rpoH* transcription, and thus ultimately an underlying factor controlling the production of HSPs remains elusive, although it may very well be that there is more than one such factor as is suggested by the complex promoter structure of *rpoH*.

A note on thermotolerance. Shifting *E. coli* from low temperature (c. 30°C) to heat-shock-inducing temperatures (42°C) endows the cells with a degree of thermotolerance to the lethal temperatures (>50°C), compared to cells not preinduced at 42°C. This protection to lethal temperature challenge is a transient effect which is at a maximum for 30 minutes after exposure to 42°C, and disappears after 1 hour (Yamamori and Yura, 1982). It was thought that the HSPs were responsible for this thermotolerance, but this is now uncertain.

VanBogelen *et al.* (1987) have reported that induction of the HSPs at 28°C by overexpressing σ-32 from an inducible promoter on a multicopy plasmid does not enhance cell viability when exposed to 50°C. Their system produced all but four of the HSPs at comparable levels to that of induction by heat. They also showed that some agents that do induce most of the HSPs can give cells some thermotolerance. A striking example is exposure to hydrogen peroxide for 60 minutes. Only the *htpG* gene product is induced by this treatment, yet these cells are thermotolerant. The *htpG* gene product is also induced to high levels by non-thermotolerance-inducing agents. The authors suggest that some other system, distinct from the heat-shock response, is involved with thermotolerance and allows resistance to thermal killing. Proteins produced by the heat-shock response allow cells to cope with mild stresses associated with normal growth. Cells which are deleted for *rpoH* and so produce no σ-32 cannot survive temperatures greater than 20°C (Zhou *et al.*, 1988), implicating vital roles for at least some of the HSPs at 'normal' physiological temperatures.

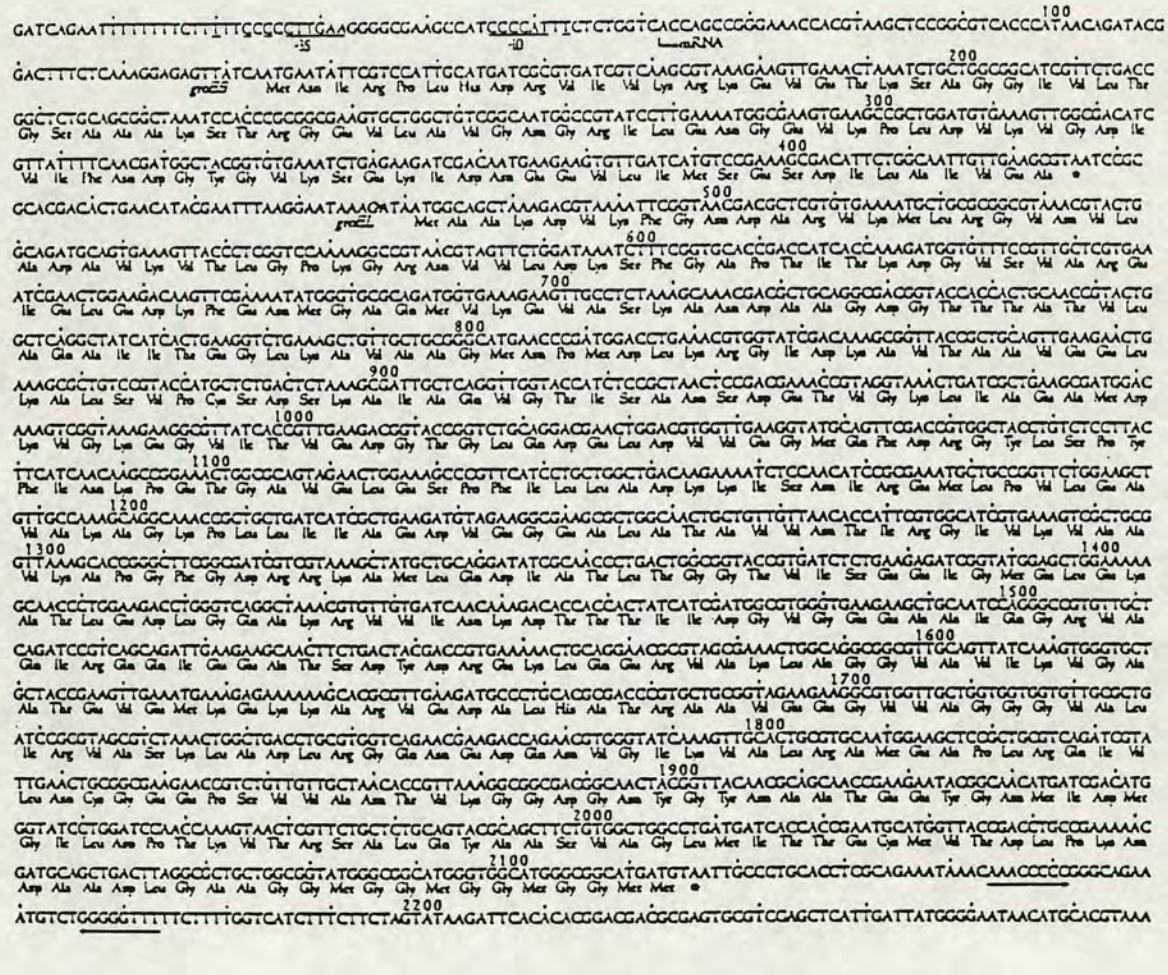
1.3 The GroE proteins — major heat-shock proteins

Heat-shock proteins of much current interest are the GroE proteins. These demonstrate the qualities of a growing protein family, 'the chaperonins', a subset of a group of proteins known collectively as 'molecular chaperones'.

The *groE* genes are located at 94 minutes on the *E. coli* K-12 genetic map and together form an operon. Deletion analysis using $\lambda groE^+$ transducing phages showed that the operon consists of two genes, *groES* and *groEL* (Tilly *et al.*, 1981). The genes reside on an 8.1 kb *Eco*RI chromosomal DNA fragment and have been sequenced (Hemmingsen *et al.*, 1988). Two transcriptional promoters have been found, a major σ-32 promoter, and a minor σ-70 about 20-25 bp downstream (Figure 1.3.1). The σ-70 promoter is potentially quite strong, but the σ-32 promoter dominates transcription in the normal temperature range (Kusukawa and Yura, 1988). Both GroES and GroEL are induced by heat-shock; GroEL can constitute greater than 10% of the total cellular protein at its maximum (Neidhardt *et al.*, 1984). *GroES* is located at the 5' end of the operon and *groEL* at the 3' end. GroES (small) is a 10 kDa polypeptide and GroEL (large) is 58 kDa. Both proteins form multimers *in vivo* with the GroES protein forming a single ring structure with between six and eight (but probably seven) members (Chandrasekhar *et al.*, 1986), and GroEL forming a large 14-membered structure comprising two seven-membered rings stacked on top of each other with dimensions of approximately 13 nm diameter and 11 nm in height, as judged by electron microscopy (Hendrix, 1979). These multimers are the functional forms of the respective proteins. GroEL exhibits a weak ATPase activity, even though it binds ATP avidly. Each GroEL tetradecamer can bind 14 molecules of ATP and half of these are hydrolysed in a co-operative manner (Bochkareva *et al.*, 1992). Mutations in either gene can lead to temperature-sensitive growth with impaired DNA and RNA synthesis and cell-division defects (Wada and Itikawa, 1984; Georgopolous and Eisen, 1974). *GroE* mutants are also unable to support the development of certain bacteriophages (see *1.4.1*). GroES and EL interact *in vitro* since GroES can partially inhibit the ATPase activity of GroEL (Chandrasekhar *et al.*, 1986), and in the

presence of ATP the GroES protein demonstrates specific retention on a GroEL affinity column. It is also believed that they interact *in vivo* since mutations in one can suppress mutations in the other (Tilly and Georgopolous, 1982). Fayet *et al.* (1989) showed that the GroE proteins are required for cellular viability at all temperatures implying that these proteins have much more crucial functions within the cell than just coping with environmental stress. However, the precise nature of these functions has until recently remained obscure.

Figure 1.3.1 Nucleotide sequence of the *groE* operon.



Nucleotide sequence of the *groE* operon, and the predicted amino acid sequences of the GroES and GroEL proteins. Taken from Hemmingsen *et al.*, 1988.

GroEL — A bacterial common antigen. Sompolinski *et al.* (1980) reported finding an antigen in *Pseudomonas aeruginosa* that was antigenically related to a similarly sized antigen in a wide range of Gram-negative bacteria including *E. coli*. For this reason it was named the 'common antigen'. Thole *et al.* (1988) used polyclonal sera and monoclonal antibodies to detect cross reactivity in Gram-negative, Gram-positive bacteria and archaebacteria. The common antigen in *E. coli* is the GroEL protein. Bacterial common antigens tend to have highly immunodominant epitopes, which have a role to play in some types of auto-immune diseases/responses. Indeed, anti-GroEL anitibodies have been found in patients with certain chronic and autoimmune diseases and in diseases caused by a wide range of pathogens (for reviews see Young *et al.*, 1988; Young and Elliott, 1989; Cohen, 1991). When the GroEL (HSP60) proteins from a wide selection of organisms are analysed, the conservation of amino acid sequence is high. Even between *E. coli* and humans the amino acid identity is nearly 50% (Jindal *et al.*, 1989). This high degree of conservation suggests that the HSP60 protein has an important basic function, vital to cellular life. In eukaryotic cells GroEL homologues are found in the mitochondria (McMullin and Hallberg, 1988). These organelles are thought to be the remnants of an ancient symbiotic relationship between the earliest form of eukaryotic cell and an invading bacterium. In the cytoplasm of eukaryotic cells no obvious HSP60s homologues have been found, but in the archaebacterium *Sulfolobus* a very abundant heat-inducible protein, which forms a particle with eight- or nine-fold symmetry, has been identified and named TF55 (Trent *et al.*, 1991). A homologue of TF55 has been identified in the cytoplasm of higher eukaryotes (which is held to have originated from the archaebacteria) and it is speculated that this protein (TCP-1) in the cytoplasm and HSP60 in the mitochondria are involved in similar cellular functions. The functional similarities between the two proteins will be discussed later (see *1.6*).

1.4 Functions of the GroE proteins

1.4.1 Phage morphogenesis

GroE mutants of *E. coli* were first isolated as strains that could not support the morphogenesis of bacteriophages λ and T4 (Takano and Kakefunda, 1972; Georgopolous *et al.*, 1972, 1973; Coppo *et al.*, 1973; Sternberg, 1973; Hendrix and Tsui, 1978; Georgopolous and Hohn, 1978; Hohn *et al.*, 1979; Revel *et al.*, 1980). Phages T5 and φ186 were also found to be affected (Zweig and Cummings, 1973; Hocking and Egan, 1982). In the case of phage λ the GroE proteins act in conjunction with the λNu3 scaffold protein (*gpE*) to promote the correct assembly of the 12-membered asymmetric ring structure of the λB head–tail connector protein (*gpB*), eventually leading to the formation of the phage prohead (Kochan and Murialdo, 1983; Kochan *et al.*, 1984). Thus in *groES*- and *groEL*-mutant strains infected with phage λ, various parts of the final phage particle are amassed but are not assembled together. In the case of phage T4 it is again the head structure of the phage particle that is not correctly assembled, but for T4 assembly, only GroEL and not GroES appear to be required (Keppel *et al.*, 1990). It has been shown that Gp31 of T4 allows the orderly assembly of the Gp23 capsid protein (Georgopolous *et al.*, 1972). In *groEL*-mutant cells the Gp23 protein is found to aggregate in amorphous 'lumps' in the bacterial plasma membrane. Mutations in both *gp23* and *gp31* can suppress the *groEL* mutation and allow development of viable viruses. Mutational analysis suggests that Gp31 is replacing the function of GroES in the GroEL–ES system. Gp31 and GroES show no sequence similarities, but they are of similar sizes (12 kDa and 10 kDa respectively) and have similar acidic pI values (Keppel *et al.*, 1990).

In the case of phage T5 and φ186 it is the tails of the virus particles that are not assembled. Suppressing mutations arise in the T5 gene *D19*, and in the φ186 gene *H* (Kochan and Murialdo, 1983; Kochan *et al.*, 1984). Taken together these results suggested that the GroE proteins of *E. coli* are involved in assisting other proteins assemble into functional structures.

Most of the mutant strains, which were isolated on the basis of their bacteriophage resistance, were also found to be temperature

sensitive (Georgopolous and Eisen, 1974). Obviously GroE proteins are not maintained in the cell to allow successful infection with phages; the mutants made it possible to analyse the strains' phenotypes at non-permissive temperatures with a view to determining *in vivo* functions. When shifted from permissive to non-permissive temperatures *groE*-mutant strains exhibit defects in several fundamental cellular processes. DNA and RNA synthesis rates are reduced (Wada and Itikawa, 1984), there is an overall decrease in generalized protease activity (Straus *et al.*, 1988), and the cell-division process is blocked, probably at an early stage, leading to the formation of long filaments (Georgopolous and Eisen, 1974). A temperature-sensitive mutation designated *fam-715*, originally isolated as a filamentous cell-division mutant, was shown to actually be an *rpoH* mutant encoding the heat-shock regulator σ-32 (Tsuchido *et al.*, 1986). Kusukawa and Yura (1988) showed that a filamentous temperature-sensitive *rpoH* deletion mutant could be suppressed by overexpression of the *groE* genes, a suppression that can be improved by concomitant overexpression of the *dnaK* gene. The fact that the *groE* gene products alone can suppress the filamentation of *rpoH* mutants suggests that they may be intimately involved with the cell-division process. Indeed, shortly after the chromosomal *groE* promoter is switched off, the cell begins to filament (A. Horwich, personal communication). It is also known that wild-type *E. coli* cells transiently filament after heat shock, which could be caused by partial denaturation of the cell-division apparatus. This effect cannot be reversed without some σ-32-inducible protein(s), of which the GroE proteins must be worthy candidates, although other heat-shock mutants, such as *dnaK*, also show blocks to division at non-permissive temperatures (Bukau and Walker, 1989a). However, like *groE* mutants, *dnaK* mutants show a variety of phenotypes at restrictive temperatures such as abnormal chromosome segregation (Bukau and Walker, 1989a); the whole picture is probably rather complicated.

1.4.2 Genetic suppression analysis

RNA polymerase. Another genetic approach to determining the roles of the GroE proteins is analysis of suppressing mutations. A mutation

in *rpoA*, the RNA polymerase α-subunit, has been shown to suppress a *groES* mutation (Wada *et al.*, 1987). RNA polymerase is a large multimeric protein and it has been suggested that the *rpoA* mutation implies a potential role for GroE proteins in polymerase assembly (Georgopolous and Ang, 1990). Alternatively, the *rpoA* mutation may result in increased expression of the *groE* operon allowing the *groES* mutation to be suppressed by overexpression of the mutant protein.

An interesting observation was made by Kashlev *et al.* (1989) when trying to determine the ratio of rifampicin-sensitive (Rif^S) to rifampicin-resistant (Rif^R) RNA polymerase β-subunits (the product of the *rpoB* gene) required to allow the cells to become Rif^R. When they overexpressed the Rif^R β-subunit on a multicopy plasmid the cells grew poorly on rifampicin, and the Rif^R RpoB subunits were found as insoluble aggregates. When the cells were heat shocked, or exposed to ethanol (another HSP-inducing agent), the cells became much more Rif^R. This suggests that certain heat-shock proteins could be involved with disaggregation of the insoluble subunits and/or assembly of these subunits into the RNA polymerase holoenzyme, which could itself have been partially denatured during the heat-shock treatment. These results do indeed implicate at least some of the heat-shock proteins in RNA synthesis. Skowyra *et al.* (1990) showed that heat-inactivated RNA polymerase could be disaggregated and rejuvenated for function by the addition of the heat-shock protein DnaK in an ATP hydrolysis-dependent fashion *in vitro*.

Single-stranded DNA-binding protein. Ruben *et al.* (1988) showed that a temperature-sensitive mutation *ssb1*, encoding the single-stranded DNA-binding protein, required for DNA replication and DNA damage repair systems, could be suppressed by a secondary mutation in the *groEL* gene (*groEL441*). This was shown to be allele specific since *groEL441* could not suppress another *ssb* mutant (*ssb113*). The Ssb protein forms a tetramer *in vivo* and the mutant Ssb1 protein can suppress the *ssb1* mutation if it is overexpressed (Chase *et al.*, 1983). It is thought that the mutant-suppressing GroEL441 protein is directing the high-temperature assembly of the functional Ssb1 protein (which tends to dissociate at high temperature) and is maybe achieving this by shifting the equilibrium between non-functional

monomers and functional tetramers to the side of the tetramers or perhaps by providing a specific scaffold (not provided by wild-type GroEL) for the correct alignment of mutant monomers that allows successful tetramer formation. More recently the same group have shown that the *ssb113* mutation can be suppressed by another allele of *groEL*, *groEL46*. Presence of the *groEL46* mutation completely reversed all the phenotypes associated with the *ssb113* mutation including temperature-sensitive growth, temperature-sensitive DNA synthesis and UV sensitivity. Conversely, strains carrying *groEL46* grow better at 42°C when combined with *ssb113* than they do in the presence of *ssb*$^+$; however, *ssb113* does not restore *groEL46*'s ability to plate λ phages. *GroEL46* does not suppress any other temperature-sensitive alleles of *ssb*. In the case of *ssb1* suppression by *groEL441* only the DNA replication defect was corrected allowing high-temperature growth, and since the defects seen with *ssb1* and *ssb113* are so markedly different, it is thought by the authors that the mechanisms of suppression also differ (Laine and Meyer, 1992).

Genetic suppression by overexpressing the groE *genes*. Other attempts to discover true GroE protein substrates have used multicopy plasmids carrying the *groE* genes to overexpress the proteins in order to suppress temperature-sensitive mutations. The suppression of *rpoH*-deletion mutations has been discussed earlier. Jenkins *et al.* (1986) and Fayet *et al.* (1986) showed that such an approach allowed high-temperature growth of some *dnaA*^{ts} mutants, and this will be covered in detail later. Van Dyke *et al.* (1989) demonstrated that overexpression of the *E. coli* GroE proteins could suppress several different temperature-sensitive histidine biosynthesis mutations in *Salmonella typhimurium* and used this as an argument to suggest that overexpression of the *groE* operon would suppress many different temperature-sensitive mutations. It should be noted in this regard, however, that *ssb* mutations, suppressible by certain *groEL* alleles, are not suppressed by overexpression of the wild-type *groE* genes, and likewise that not all *dnaA*^{ts} alleles are suppressed by GroE overexpression. Interestingly Van Dyke *et al.* found that mutant proteins which function as monomers were not suppressed

suggesting that GroE proteins are acting as multimeric assembly factors, rather than single polypeptide folding factors.

The ams *gene*. As a slight digression I would like to tell the tale of *groE* and the *ams* gene. *Ams*^{ts} mutants exhibit altered mRNA stability with mRNA half-life increasing approximately 5–10 fold at non-permissive temperatures (Kuwano *et al.*, 1977). An attempt to clone the *ams* gene product utilized a strategy involving transformation of the *ams*^{ts} strain at the non-permissive temperature with a plasmid-borne *Eco*RI *E. coli* chromosomal DNA library. A complementing DNA clone was obtained, and this was subcloned to give the smallest possible complementing DNA fragment. This was sequenced and published as the sequence of the *ams* gene (Chanda *et al.*, 1985). It later transpired that what had been sequenced was an internal fragment of the *groEL* gene, and the originally obtained complementing clone carried the whole *groE* operon. It seemed that overexpression of the *groE* genes could suppress the *ams* mutation, and that this could even be achieved with only a small 10 kDa fragment of the GroEL protein. Subsequent work by Claverie-Martin *et al.* (1991) has shown that the *ams* gene product is a large 110 kDa protein, which acts as a ribonuclease, and is known as RNase E. Other attempts were also undertaken to repeat the results of Chanda *et al.* but no suppression was seen, even with the whole *groE* operon. Work in our laboratory has come to the same conclusion. However, recent attempts to purify functional RNase E have resulted in purification of a large complex with an associated RNase E activity, and the major component of this complex is GroEL (Sohlberg *et al.*, 1993). It now seems quite possible that RNase E and GroEL interact *in vivo*, and overexpression of *groE* may well suppress *ams*^{ts} mutations under certain circumstances.

Fayet *et al.* (1989) showed that both of the GroE proteins are essential for viability at all growth temperatures (18–42°C) and so the importance of the GroE proteins cannot be understated, but what are the proteins actually doing? The bacteriophage work and the suppressor analysis suggest that GroE proteins are involved in

assembling multimeric protein structures, and the analysis of the mutants showed major disruption to various cellular processes. It is now generally accepted that the GroEL protein (and all the HSP60 homologues) act in conjunction with GroES as a 'molecular chaperone'.

1.4.3 The GroE proteins are molecular chaperones

The term 'molecular chaperone' was first used to describe the role of nucleoplasmin, a protein involved with assembling DNA and histones isolated from *Xenopus* oocytes into ordered nucleosomes (Laskey and Earnshaw, 1980). Without the nucleoplasmin the DNA and histones formed an unorganized aggregate *in vitro*. John Ellis of the University of Warwick adopted the term to describe a protein of interest to him associated with rubisco (ribulose-1-5-bisphosphate carboxylase oxygenase), an enzyme involved with fixing CO_2 in higher plant chloroplasts. Rubisco can amount to as much as 50% of a plants total cell protein and is therefore probably the most abundant protein on earth. In higher plants, rubisco is a multimer consisting of eight small and eight large subunits. The large subunits are encoded by plastid genes and the small by nuclear genes and thus are imported from the cytoplasm. For attainment of the correct rubisco conformation, another protein called the rubisco subunit-binding protein (RSBP) is required. RSBP does not form part of the final rubisco enzyme, but is required during assembly of the large subunit octamer from four dimers (Barraclough and Ellis, 1980). Ellis called his RSBP a molecular chaperone and showed that it was made up of two types of subunit (α and β) found in equal amounts. The α and β subunits showed 50% sequence similarity to each other and by scanning a data base for sequence similarities with the α subunit found the *groEL* gene of *E. coli*. GroEL shares around 50% sequence similarity with RSBP (Hemmingsen *et al.*, 1988). RSBP was also shown to have the same tetradecameric ring structure and sevenfold symmetry as GroEL. Attempts to express functional higher plant rubisco in *E. coli* have failed, but assembly of dimeric and octameric prokaryotic forms of rubisco in *E. coli* has been demonstrated (Gatenby, 1988; Goloubinoff *et al.*, 1989b). Dimerization of the large subunits seems to be a crucial step in the assembly of these prokaryotic form of rubisco.

It has been shown that this step requires the function of both GroE proteins, and so it is proposed that the GroEL protein is acting in place of RSBP in this system (Goloubinoff *et al.*, 1989b). The requirement for a GroES-like protein in the formation of higher plant rubisco has not been demonstrated, but GroES-like proteins have been found in barley chloroplasts and mitochondria, and their involvement in the reaction seems plausible (Hartman *et al.*, 1992).

1.4.4 The role of molecular chaperones

The original definition of a molecular chaperone was a protein that (like a human chaperone) brings potentially complementary surfaces together, but makes sure that no improper or premature interactions occur, thus allowing a correct liaison without actually conveying any steric information about that liaison. Ellis' adoption of the term 'molecular chaperone' to explain the action of RSBP has now been extended to cover a growing list of different proteins that bind polypeptides and mediate their folding and/or assembly. Ellis proposed that molecular chaperones of the GroEL/HSP60-type should be designated 'the chaperonins', which seems more appropriate than terminology that implies their involvement solely during heat-shock (see Ellis and Hemmingsen, 1992; Ellis and Van der Vies, 1991; Georgopolous, 1992; Hartl and Martin, 1992; Ellis, 1993;). Recently the HSP60 class of chaperonins has been divided into two sub-classes; (i) HSP60s, and (ii) TCP-1-like proteins (see *1.6*). It is still uncertain whether molecular chaperones of the HSP60 type actually assemble multimeric structures, or promote the correct folding of unstructured monomers, or both. *In vitro* studies seem to favour the second possibility and have shown that the GroEL protein binds unfolded polypeptides and gives them an environment conducive to a correct folding pathway. It was a long-held belief that all the information required for a polypeptide to assume its final folded state lay within the amino acid sequence of that polypeptide. The early protein renaturation experiments demonstrated this (Anfinsen and Scheraga, 1975). However, the cellular environment is vastly different from that found in a test-tube and it now seems that at least some proteins do need help to realise their final structures. Many proteins seem to be able to fold-up correctly unaided *in vitro* when the correct

conditions are supplied and therefore possess all the necessary steric information for folding within their amino acid sequences, but *in vivo* they might well require molecular chaperones to prevent misfolding and aggregation. If this is the case then they may be thought of as active in binding unfolded polypeptides, but passive in directing their folding.

In vitro *studies*. The first *in vitro* demonstration of polypeptide binding by GroEL was a superb piece of work by Bochkareva *et al.* (1988). They showed that *E. coli* GroEL protein could bind newly translated, unfolded pre-β-lactamase (pre-β-lac) and chloramphenicol acetyltransferase (CAT), both of which need to be in an unfolded conformation in order to traverse the bacterial plasma membrane into the periplasmic space. A transcription/translation system was used to produce the pre-β-lac and CAT polypeptides. An N-acyl-Met-tRNA^{met} with the acyl group containing a precursor to a highly photoreactive carbene radical was used to add the initial N-terminal methionine residue, resulting in polypeptides that are labelled at one site only. The *E. coli* translation extract (S30) contains GroEL tetradecamers but these could be removed (up to 95%) by passing the extract through an anti-GroEL sephadex affinity column. Exogenous GroEL was then added to the translation mix. Photoactivation led to the pre-β-lac/CAT polypeptides binding to the GroEL tetradecamers. Furthermore, it was shown that the protein was binding only when in an unfolded state. Addition of Mg–ATP, but not a non-hydrolysable analogue, to the GroEL–polypeptide complex resulted in dissociation of the polypeptide, indicating a role for GroEL's weak ATPase activity. Under these dissociating conditions the pre-β-lac polypeptide was found to be translocated into isolated inverted membrane vesicles. Photoactivation with the additional presence of GroES led to binding between pre-β-lac and GroES, but only in the presence of GroEL. The authors use this to argue that GroES and newly synthesized pre-β-lac are closely associated only when pre-β-lac is bound to the surface of GroEL (Bochkareva and Girshovich, 1992)

A more detailed study of the interaction of pre-β-lac and GroEL was conducted by Zhan and Plückthun (1992). They show that an early folding intermediate of pre-β-lac is recognized by GroEL and so

prevented from aggregating. The form that is discharged from GroEL by Mg–ATP hydrolysis is another folding intermediate, which can misfold to a non-native conformation (but not into an aggregate) in a pH-dependent fashion. This intermediate is not bound by GroEL and would fold correctly under normal physiological conditions without any external assistance. They therefore suggest that GroEL prevents aggregation by binding polypeptides that have the capacity to aggregate, and processing the polypeptides to conformations that will not aggregate. Interestingly they also showed that mature-β-lac does not bind to GroEL under any folding conditions and so propose that the N-terminal signal sequence is a motif recognized by GroEL.

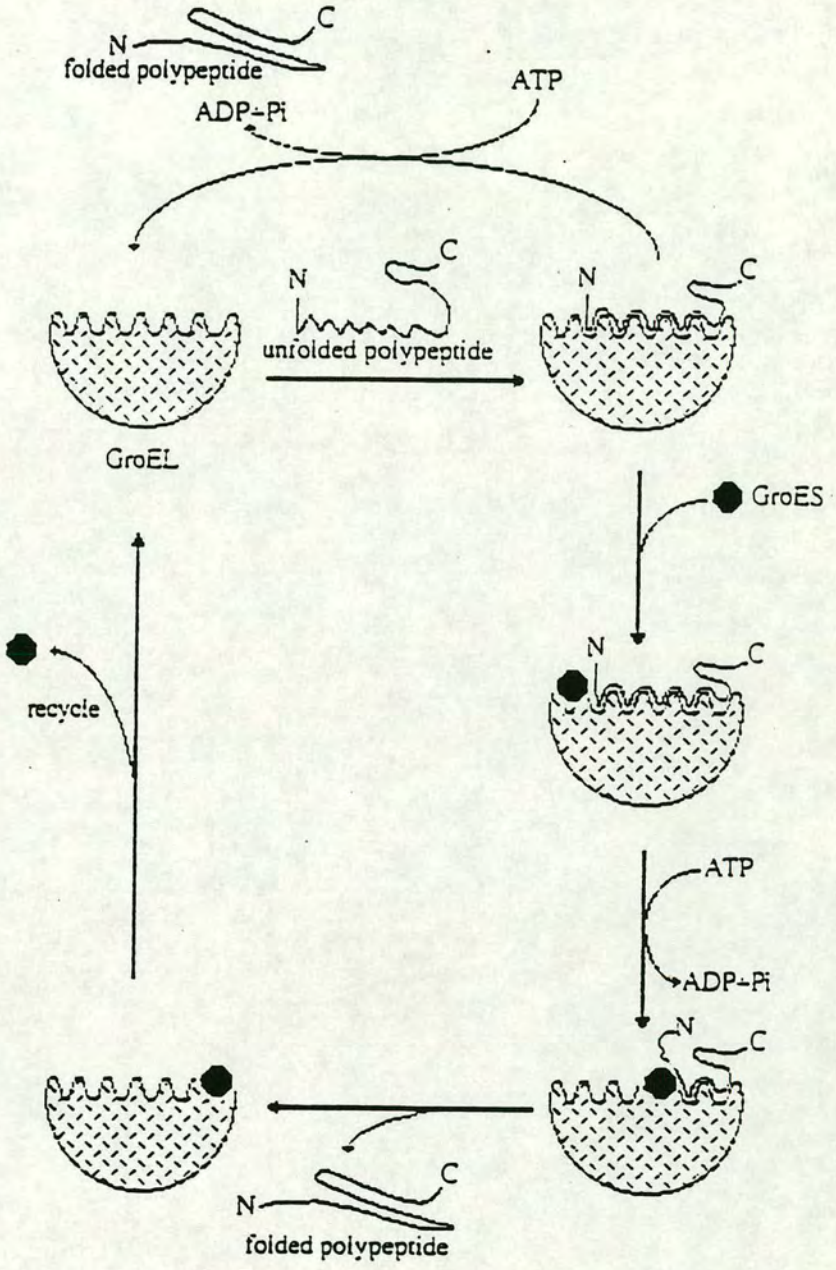
The binding of unfolded polypeptides by the GroEL tetradecamers seems consistent with its proposed role in the heat-shocked cell, in that thermally denatured proteins are being sequestered and thus prevented from aggregating with GroEL soaking-up the denatured proteins like a 'molecular sponge' (LaRossa and Van Dyke, 1991). However, the work of Bochkareva *et al.* (1988) also suggests a role for the GroEL protein in membrane translocation of proteins, and indeed Kusukawa *et al.* (1989) have shown that the export of pre-β-lac is defective in *groE*^{ts} mutants *in vivo*, although protein export is not yet thought to be a major function of the GroE proteins and other specialized export systems are normally employed (reviewed by Kumamoto, 1991).

Several reports have demonstrated the formation of complexes between unfolded or denatured polypeptides and GroEL. (Goloubinoff *et al.*, 1989a; Viitanin *et al.*, 1990; Buchner *et al.*,1991; Mendoza *et al.*, 1991, 1992; Evers *et al.*, 1992). Rather than go into each case individually, I will attempt to generalize about the results. In most cases purified substrate proteins were denatured in urea or guanidine–HCl and diluted into denaturant-free buffers at concentrations that would normally lead to aggregation of the protein. In the presence of GroEL the denatured proteins would be found complexed to the GroEL with one (or possibly two) unfolded monomers per GroEL tetradecamer. Addition of Mg–ATP to the complex often led to release of the substrate polypeptide and production of a functional, and therefore a correctly folded, protein. Mg–ATP alone, however, was not enough to cause dissociation of the

bound peptide in some cases and the addition of GroES protein was required for release. It is believed that different proteins will have different binding characteristics with GroEL and some require the additional 'push' from GroES in order to fold and be released. The polypeptides are presumably leaving GroEL in a controlled manner conducive to correct folding and it has been suggested that for the folding of some proteins the presence of GroES is required in order to participate in a critical folding step rather than simply accelerating the folding process. This has led to a model of GroEL/GroES function (the 'cog-wheel model') proposed by Georgopolous (1992) (Figure 1.4.1). All of the above work was performed using *E. coli* GroEL and non-*E. coli* substrate proteins.

Obviously GroEL has not been maintained in *E. coli* so that molecular biologists could perform *in vitro* experiments, and work done using proteins found in *E. coli* have proved interesting.

Figure 1.4.1 The cog-wheel model of GroEL/GroES protein folding.



The cog-wheel model of GroEL/GroES protein folding. The GroEL tetradecamer binds unfolded or partially folded substrate polypeptides and can (i) release them following nucleotide binding/hydrolysis (upper part of the cycle), or (ii) release them following nucleotide binding/hydrolysis and interaction with GroES (lower part of cycle). Adapted from Georgopolous, 1992.

In vitro *studies using* E. coli *proteins*. Lissin *et al.* (1990) examined the assembly of the GroEL tetradecamer itself *in vitro*. It was shown that GroEL tetradecamers could be formed from GroEL monomers in the presence of ATP and Mg^{2+} ions at 23°C (but not on ice). Assembly intermediates were not found, suggesting that the assembly of the GroEL tetradecamer is highly co-operative. Assembly of the GroEL monomers was also found to be enhanced by the presence of GroEL tetradecamers. The authors refer to this as 'self-chaperoning'. Ziegelhoffer has shown that the λ phage λB head–tail connector protein monomers, (the protein that is not assembled into its final dodecameric ring structure in *groE* mutants), bind GroEL *in vitro* and the complex is dissociated on addition of ATP. However the dodecameric ring structure of λB protein was not formed, not even in the presence of GroES and the λNu3 scaffold protein (cited in Zeilstra-Ryalls *et al.*, 1991b); a result which possibly casts doubt on GroEL's ability to assemble multimeric structures. This is countered by reports in which the formation of dodecameric glutamine synthetase (GS) from *E. coli* is promoted by the presence of GroEL and Mg–ATP although the author argues that ATP hydrolysis is not absolutely required for the dissociation of bound GS monomers (Fisher, 1992, 1993). Mizobata *et al.* (1992) have shown that *E. coli* tryptophanase can be folded *in vitro* in the presence of GroEL and GroES. More significantly, they argue that ATP hydrolysis is not required for this refolding to take place, but merely nucleotide binding (either ATP or ADP) by GroEL allows the folding reaction to proceed. Fong and Bridger (1992) have shown that heterotetrameric succinyl-CoA synthetase (SCS), when denatured can reassemble to an active conformation without any requirement for GroEL or other molecular chaperones. Moreover, they show that the unfolded polypeptides do not form complexes with the GroEL tetradecamers, and that SCS production in *groE* mutant bacteria is unaffected at restrictive temperatures. It could be that the monomers are folding up too quickly for the GroEL molecules to bind them, or that GroEL is selective and that something is missing from the SCS polypeptides which is common to all the other substrate proteins tested so far.

An attempt to determine how many *E. coli* proteins could bind to GroEL was performed by Viitanin *et al.* (1992). Here they used a

soluble protein cell extract labelled with [S^{35}]-methionine and denatured in 5 M guanidine–HCl, to show that about half of the unfolded protein complex could be bound to GroEL tetradecamers and subsequently released on addition of Mg–ATP. This rather crude analysis showed that a multitude of denatured *E. coli* proteins have the potential to bind to GroEL, but did not attempt to address the question of which proteins are bound and which are not. The authors state that presumably several folding intermediates for each protein are present in the protein complex some of which would be conformations not recognized by GroEL, and thus differentiation between bound and unbound would be impossible.

In vitro analysis of purified GroEL from a *groEL140* temperature-sensitive, bacteriophage λ-resistant *E. coli* mutant has shown that this form of the protein is 1.5 times slower than the wild-type protein at hydrolysing ATP, is more sensitive to trypsin digestion, and releases bound polypeptides six to seven times slower, possibly owing to a poor interaction with GroES (Baneyx and Gatenby, 1992). The authors also report that non-native rubisco remains associated two times longer with GroEL140 compared to wild-type *in vivo*.

Landry and Gierasch (1991a, b) observed that the experimental *in vitro* GroEL substrates rubisco, β-lactamase, citrate synthase and rhodanese all contain solvent-exposed amphipathic amino-terminal α-helices and suggested that it was this secondary structure, or its potential, that was recognized by GroEL. However, Schmidt and Buchner (1992) have reported that a protein fragment consisting entirely of β-sheet secondary structure elements is bound and released by the GroEL–GroES–ATP system *in vitro*. It seems probable that the interaction of GroEL with a protein substrate results from the nature of that protein's early folding intermediates and not on specific elements of secondary structure. It is now generally accepted that GroEL tetradecamers interact with polypeptides that are essentially unfolded but still contain a reasonable amount of secondary structure. It has been proposed that this level of structure is energetically favourable compared to other folding intermediates and has a flexible tertiary structure with a dynamic hydrophobic core as described for the 'molten-globular state' (Martin *et al.*, 1991). (For a review on the molten-globular state see Kuwajima, 1989.) The work,

which suggests that GroEL is interacting with molten globules, has been based on tryptophan fluorescence spectroscopy and relies on the fact that GroEL contains no tryptophan residues. Some controversy has arisen, however, because it has recently been suggested that the original GroEL sequence is wrong and amino acid analysis of purified GroEL demonstrates the presence of one tryptophan residue (Price *et al.*,1991). However, Hayer-Hartl and Hartl (1993) argue that the background tryptophan fluorescence seen in purified GroEL preparations is caused by contaminating proteins in the sample, which are present owing to GroEL's polypeptide-binding capacity. The majority of researchers in the field agree that GroEL does not contain a tryptophan residue.

There is a report that some 'fine-tuning' occurs with the GroEL protein, altering its capacity to bind polypeptides. When cells are subjected to heat shock a subset of the GroEL protein becomes covalently modified. The modification that occurs is probably phosphorylation and is reversible. The phosphorylated form of GroEL releases bound polypeptides on ATP hydrolysis more easily than the normal form of GroEL (Sherman and Goldberg, 1992). It is proposed that the phosphorylated form has less of a requirement for GroES than the normal form. This maybe important for GroEL function during the stress response.

Recent *in vitro* work has addressed the question of what are the functional domains within the GroE proteins. Two separate mutations in the amino-terminal region of GroEL have been shown to prevent assembly of the tetradecameric form of GroEL (Horovitz *et al.*, 1993a, b). Both mutants had amino acid substitutions at positions highly conserved within the HSP60 protein family, and thus implicate this region of the protein as essential for the assembly of the GroEL tetradecamer from monomers. Complementary work by Burns *et al.* (1992) and Landry *et al.* (1993) has demonstrated a region of GroEL required for interaction with GroES, and a region of GroES required for interaction with GroEL, respectively. The regions of GroEL tetradecamers that bind GroES are thought to be at the ends of the GroEL cylinder. Electron microscopy suggests that GroES binds one end of the GroEL tetradecamer and induces a structural change, so that the other end of the GroEL molecule is not available for GroES

interaction (Langer *et al.*, 1992b; H. Saibil, personal communication). The region of GroES required for interaction with GroEL is a stretch of 15 amino acids, which are thought to form an accessible mobile loop. Out of eight *groES* mutants, all were found to contain mutations affecting this region and so it seems to be functionally important. The loops lose mobility and become stabilized when complexed with GroEL (as judged by NMR), and it is believed that the seven mobile loops of a GroES heptamer are interacting with sites on seven of the GroEL subunits. There has been much speculation about where on GroEL the polypeptide-binding site(s) are located. Braig *et al.* (1993), using gold-labelled dihydrofolate reductase, have demonstrated peptide binding within the central hole of GroEL and suggest a 90 kDa capacity for this space. Using electron microscopy, Langer *et al.* (1992b) have also visualized polypeptides bound within GroEL's central cavity. This result, along with most of the other *in vitro* data concerning GroEL's chaperoning activity, suggest that the GroEL protein may be more involved in the folding of monomeric polypeptides rather than the assembly of multimeric protein structures. It seems feasible that the multimeric structures, which are not assembled in *groE* mutants, arise as a result of incorrect folding of individual subunits of that structure. Perhaps there are other factors within the cell involved in multimeric assembly which are indirectly dependent upon functional GroE complexes.

In vivo *studies*. Demonstration of the formation of complexes between certain proteins and GroEL in *E. coli* has been reported by several groups (Landry and Barlett, 1989; Govezensky *et al.*, 1991; Sherman and Goldberg, 1991; Carrillo *et al.*, 1992; Dolan and Greenberg, 1992; Lee and Olins, 1992; Wynn *et al.*, 1992). Again proteins foreign to *E. coli* have been used in these experiments, leaving us to wonder at what the native GroE substrates might be. Most of this work has shown that the protein of interest would not be functional and/or assembled in *groE*-mutant bacteria. More interestingly it was shown that proteins that normally become insoluble aggregates when overexpressed in *E. coli* can be largely solubilized by concomitant overexpression of the *groE* genes. These results suggest that the GroE proteins are not highly selective in their choice of substrate and will

help totally foreign proteins to assemble correctly. This could be very useful for researchers trying to obtain functional foreign proteins from genes cloned in *E. coli*. It seems quite possible that the prevention of protein aggregation is a major function of the GroE proteins. Proteins can be damaged by increasing temperatures, leading to aggregation and insolubility of those proteins (Pinto *et al.*, 1991). Gragerov *et al.* (1991; 1992) have shown that temperature-sensitive *rpoH* strains exhibit severe protein aggregation at restrictive temperatures, and that this aggregation can be prevented by overproduction of either GroES and GroEL, or DnaK and DnaJ. Martin *et al.* (1992) have also demonstrated that thermal aggregation of polypeptides is prevented, at least in part, by the GroE proteins.

The *groE* gene products have been found to be induced by UV irradiation and nalidixic acid in an *rpoH*-dependent fashion (Kruger and Walker, 1984) and have also been shown to be involved in the subsequent SOS repair (Liu and Tessman, 1990). Donnelly and Walker (1989, 1992) have shown that *groE* mutants are defective in *umuCD*-dependent UV mutagenesis. The products of the *umuCD* genes are thought to enable DNA polymerase III to bypass UV-induced DNA lesions. It is a processed form of UmuD protein (UmuD') that interacts with the UmuC protein to give the functional complex and it is suggested that GroE proteins protect UmuC from proteolysis while UmuD is being processed. The half-life of UmuC is reduced in *groE*-mutant strains. A demonstration of co-immunoprecipitation of UmuC with anti-GroEL antibodies suggests that an *in vivo* relationship between the two proteins is occurring.

Very recently there has been an excellent report on the role of GroE proteins *in vivo* utilizing some clever bacterial genetics. Horwich *et al.* (1993) made a *groEL* mutant that was severly temperature-sensitive, and therefore unable to form colonies above 35°C. Using this mutant they were able to demonstrate the loss of solubility of 16 diverse polypeptides on 2-D protein gels, of which three were identified and found to be citrate synthase, ketogluterate dehydrogenase and polynucleotide phosphorylase. Overall, 30% of all the polypeptides were affected by the loss of GroEL function in the mutant cells at restrictive temperatures. These results suggest that

there is a specific set of proteins that rely on GroEL to allow their folding to the native conformation.

It can be seen that the *in vivo* role of the GroE proteins are likely to be twofold; to ensure that newly synthesized proteins reach their functional conformations, and second, to prevent protein aggregation when the cell is challenged by environmental stresses. There is also a potential for the GroE proteins to be involved in the secretion of specific proteins (Kusukawa *et al.*, 1989).

1.5 Mitochondrial HSP60

The eukaryotic mitochondrion is a membrane-bound organelle, and the site of several important metabolic pathways, the most notable being oxidative phosphorylation. Mitochondria possess their own genomes but most mitochondrial-associated proteins are actually encoded by nuclear genes, and subsequently imported into the organelle. Indeed some yeast cells can survive under certain conditions without mitochondrial DNA, but cells cannot survive without mitochondria, indicating the essential nature of some proteins that are imported into the mitochondria and their role within its confines.

A eukaryotic GroEL homologue was first seen as a heat-inducible ribosome-associated protein in *Tetrahymena thermophila*. Antibodies raised against this 58 kDa protein showed that the protein was located in the mitochondria. Further analysis involving antibody cross-reactivity showed that the protein was indeed an HSP60 homologue (McMullin and Hallberg, 1988). HSP60 from *Saccharomyces cerevisiae* was cloned and sequenced, and shown to share 54% sequence identity with *E. coli* GroEL (Reading *et al.*, 1989). Like *E. coli* GroEL, the protein demonstrates sevenfold rotational symmetry, exists as a homomeric tetradecamer, is induced by heat-shock (although not as strongly as in *E. coli*) and is essential for cellular viability at all temperatures (Reading *et al.*, 1989; Hallberg, 1990). Mitochondria are thought to be the remnants of a symbiotic relationship between a bacterium and the earliest eukaryotic cell and the finding of GroEL-related proteins solely in mitochondria (and chloroplasts in higher plants) seems to confirm this theory. Closer sequence analysis suggests that mitochondria are derived from a member of the purple bacteria, and chloroplasts from the cyanobacterial lineage (Gupta *et al.*, 1989).

Role of HSP60 in the mitochondria. It could be suggested that the HSP60 in mitochondria is acting in the same way as GroEL in bacteria. That is, mediating protein folding and/or multimeric assembly pathways. An *hsp60* temperature-sensitive mutant of yeast (originally known as *mif-4*) has been shown to fail to assemble

mitochondrial ornithine transcarbamoylase even though the polypeptides that make up the functional trimeric enzyme are imported into the mitochondrial matrix. Other multimeric enzyme assemblies are also affected in *mif-4* cells, including the HSP60 tetradecamers themselves (Cheng *et al.*, 1989). It can be seen that it is likely that HSP60 functions within the mitochondria in a similar fashion to GroEL in the bacterial cell with respect to protein folding. However, the mitochondrial HSP60 proteins have also been implicated in the process of protein import (a process unknown in bacteria). The import of proteins into the mitochondria is a complex process involving several factors. Mitochondria have an outer membrane, inner membrane and internal matrix. Between the two membranes there is an intermembrane space, and regions where the two membranes come together are known as contact sites. These are the points at which imported polypeptides are translocated into the organelle (cited in Hallberg, 1990). For a protein to cross a membrane it must be in an open conformation. A current model for protein import into the mitochondria suggests that a translocated protein is bound by mitochondrial HSP70 as it emerges from the inside of the membrane, in order to keep it in an open conformation, and then the peptide is bound by HSP60, where correct folding can occur (Ostermann *et al.*, 1989; Endo, 1991; Manning-Krieg *et al.*, 1991; Pfanner *et al.*, 1992). Of course this is probably a vast over simplification of the situation and many other protein factors could be involved. This process can be compared with the model describing the sequential action of DnaK and GroE proteins (and other HSPs such as DnaJ) as polypeptides as they emerge from ribosomes in *E. coli* (Langer *et al.*, 1992a). As with GroEL, release of bound polypeptides is mediated by Mg–ATP. In *E. coli* GroE proteins have been implicated in secretion of certain proteins into the periplasmic space, and similarly HSP60 molecules in mitochondria maintain certain polypeptides in an unfolded state so that they can be exported back into the intermembrane space (Koll *et al.*, 1992). However, this is challenged by a report from Hallberg *et al.* (1993). They found that in yeast cells with HSP60-depleted mitochondria, mitochondrial matrix proteins are imported into the matrix, but are recovered as insoluble aggregates. In contrast, intermembrane-space-located polypeptides were found

correctly processed and compartmentalized. This suggests that these proteins do not enter the mitochondrial matrix during their import. Perhaps different intermembrane-targeted proteins have different importation pathways.

In chloroplasts, the cellular organelle in plants involved in photosynthesis, the GroEL homologue is the rubisco subunit-binding protein mentioned previously. It has been shown that this protein binds several imported polypeptides and its substrate spectrum is not solely rubisco subunits (Lubben *et al.*, 1989). Again HSP60 molecules are functioning in similar ways and the parallels between mitochondrial HSP60, bacterial GroEL and chloroplast rubisco subunit-binding protein are quite plain.

1.6 The TCP-1 chaperonins

Eukaryotic GroEL-homologues are found in cellular organelles. This has led to a search for molecular chaperones in the eukaryotic cytosol. A clue to finding such molecules was the observation that certain archaebacteria contain a heat-inducible protein with a familiar structure. This protein is TF55, which forms large particles consisting of a double-ring structure with eight or nine subunits per ring (Trent *et al.*, 1991, Phipps *et al.*, 1991). Like GroEL, TF55 has also been shown to bind non-native proteins and has an ATPase activity, although *in vitro* protein folding and a GroES-like co-chaperone have not yet been demonstrated.

Despite the similarity between TF55 and GroEL-homologues at the quaternary-structure level, there is little sequence similarity between the proteins. However, TF55 shares 40% sequence identity with a eukaryotic protein *t*-complex polypeptide-1 (TCP-1) (Gupta, 1990; Trent *et al.*, 1991). TCP-1 is found in all eukaryotic cell types examined so far, except mature sperm, and in yeast it is an essential gene product (Ursic and Culbertson, 1991). In higher eukaryotic cells TCP-1 is found in heteromeric particles of six to seven protein species, but the quaternary structure is still similar to TF55 (Frydman *et al.*, 1992; Lewis *et al.*, 1992). Owing to the similarities between TCP-1 and GroEL it has been proposed that TCP-1 should be a member of the chaperonin class of molecular chaperones (Ellis, 1992). Ursic and Culbertson (1991) showed that cells with a disrupted TCP-1 gene are inviable, but a cold-sensitive suppressor of the mutation was obtained. This mutant demonstrated retardation of nuclear segregation and abnormal staining of microtubules, suggesting a role for TCP-1 in microtubule-mediated processes. This observation is substantiated by the *in vitro* observations of Yaffe *et al.* (1992). They observed newly translated tubulin subunits transiently associating with the TCP-1 complex, with release from the particles being ATP dependent. It is, therefore, speculated that TCP-1 is involved in tubulin biogenesis. It is reasonable to suggest that other TCP-1 substrates will soon be found as our understanding of this latest chaperonin grows.

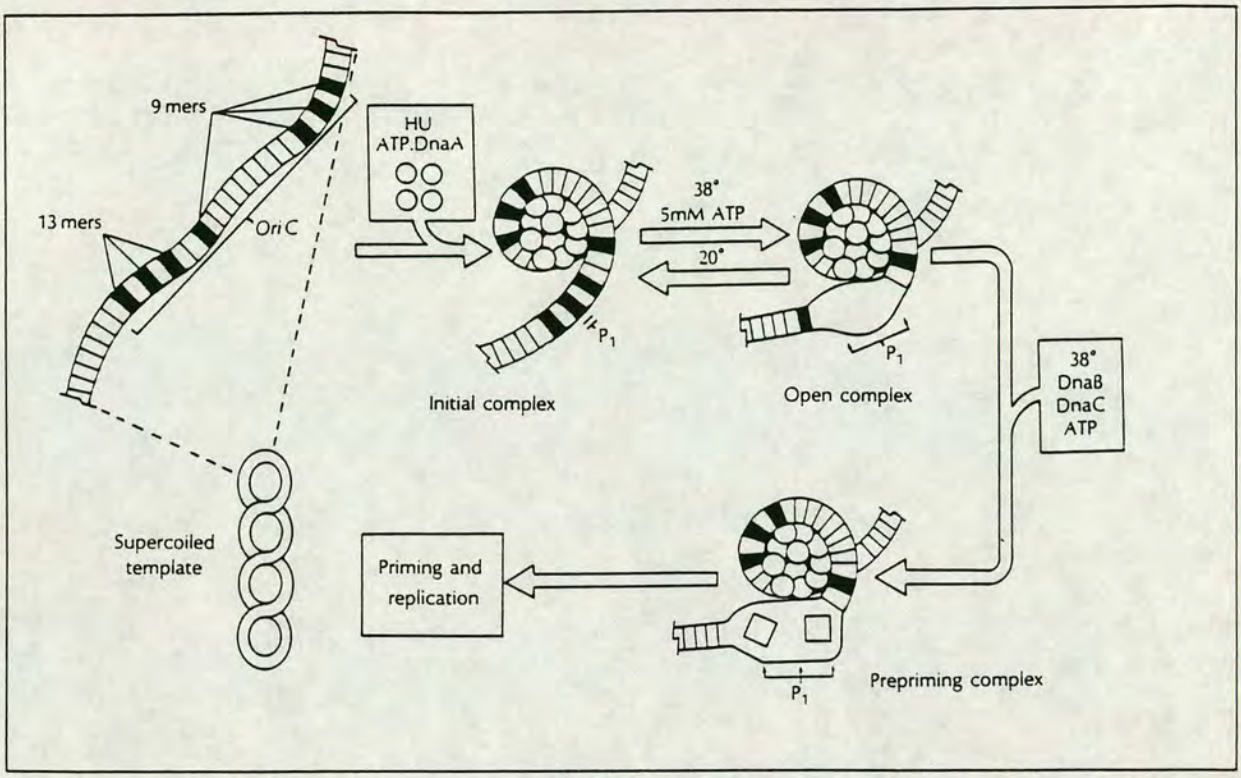
1.7 GroE proteins and DnaA

1.7.1 The DnaA protein

The DnaA protein of *E. coli* is primarily involved in the initiation of chromosomal DNA replication from *oriC*, an event that is dependent on the protein (for reviews see Von Meyenberg and Hansen, 1987; Atlung *et al.*, 1985b; Georgopolous 1989; Masters, 1989). DnaA protein binds specifically to certain sites on the DNA (DnaA boxes) that are found throughout the chromosome. At *oriC* there are five of these boxes (9-mers); when the DnaA protein is bound to these boxes, a structure is formed which has 225 bp of the *oriC* DNA wound around an aggregate of 20–40 DnaA protein molecules (Kaguni and Kornberg, 1984). When the DnaB and DnaC proteins are present, a larger complex is formed utilizing another 55 bp of the *oriC* DNA. This region of *oriC* contains three tandemly repeated AT-rich 13-mers, which become sensitive to the single-stranded nuclease P1, suggesting the formation of an 'open complex' that is stabilized by interaction with the DnaB–C proteins (Bramhill and Kornberg, 1988). The DnaC protein dissociates from the complex and DnaB helicase is then primed for its role in unwinding the DNA duplex (Figure 1.7.1).

The DnaA protein is required not only for initiation from *oriC*; many plasmid origins also contain DnaA boxes. Plasmids P1, F and R100-1 have all been shown to replicate in *dnaA*$^{+}$ and *dnaA*^{ts} hosts, but not in *dnaA*-null strains (Nagata *et al.*, 1988). Closer analysis of plasmid F's requirement for DnaA protein has demonstrated that *oriF* and *oriC* respond differently to particular mutant alleles of *dnaA*, suggesting that the two origins interact with different domains of the DnaA protein (Kogoma and Kline, 1987).

Figure 1.7.1 The role of DnaA protein in the initiation of chromosomal DNA replication at *oriC*.



A scheme for initiation at *oriC*. The DnaA protein binds the four 9-mers, organizing *oriC* around a protein core to form the initial complex. The three 13-mers are then melted resulting in the open complex. DnaB–DnaC complex can now extent the duplex opening and generate a prepriming complex, which unwinds the template for priming and replication. Taken from Masters, 1992.

DnaA protein binds ATP and ADP avidly, hydrolysing the former to the latter. DnaA–ATP is thought to be the replication competent form of the protein, while DnaA–ADP is inert with respect to DNA replication and is also very stable (Sekimizu *et al.*, 1988a). It is possible that the conversion of the ADP-bound form to the ATP-bound form could be a controlling event in the timing of DNA replication. The membrane lipid cardiolipin can stimulate the release of ADP from DnaA (Sekimizu and Kornberg, 1988), as can cAMP (Hughes *et al.*, 1988). Isolated DnaA protein is often found in aggregates complexed to phospholipids and its membrane association is thought to be important for chromosomal replication *in vivo* (Sekimizu *et al.*, 1988b). Such aggregates (in which the DnaA protein molecules are inactive for replication *in vitro*) can be converted to replication-competent DnaA by

incubation with either phospholipase or DnaK protein (Sakakibara, 1988; Hwang *et al.*, 1990).

The DnaA protein has also been shown to be involved in gene regulation, acting as a transcriptional repressor or transcriptional terminator (Schaefer and Messer, 1988; 1989). There are thought to be almost 300 potential DnaA-binding sites on the *E. coli* chromosome, of which about 90 conform to the most stringent definition of the DnaA-box sequence (cited in Masters *et al.*, 1989). The percentage that are active with respect to controlling some aspect of gene expression is unknown, but several examples of DnaA protein-mediated gene regulation have been reported. DnaA protein is believed to be self-regulating; a DnaA-box is located between the two *dnaA* promoters and is capable of repressing transcription when DnaA is bound (Atlung *et al.*,1985a; Braun *et al.*, 1985). As has been stated previously, DnaA has a regulatory effect on transcription of *rpoH* (the σ-32 gene), although the very complex promoter system of *rpoH* is bound to be regulated by several other factors too. The *guaAB* operon (encoding proteins involved in guanine synthesis) contains two DnaA-boxes and is repressed by overexpression of the *dnaA* gene, and derepressed when an *oriC*-containing plasmid is present to sequester free DnaA protein. The operon is also derepressed when a $dnaA^{ts}$ strain is shifted to non-permissive temperatures (Tesfa-Selase and Drabble, 1992). Work by Masters *et al.* (1989) has suggested that transcription of the *ftsQAZ* cell-division genes in the two-minute region of the chromosome (which carries three DnaA-boxes) responds to variations in the level of DnaA protein. Here, however, it was also shown that the *fts* genes seemed to respond to other blocks to replication initiation at *oriC*, suggesting that the derepression observed may be only indirectly related to levels of DnaA protein. Schaefer and Messer (1989) demonstrated that DnaA-boxes can act as transcriptional terminators irrespective of whether the DNA in which they lie is in a coding sequence, but are active in only one orientation on the non-template strand of the DNA, suggesting that DnaA binds DNA in an asymmetric fashion and contacts only one strand of the duplex. It now seems, however, that this work may be flawed and Messer currently believes that DnaA can act as a terminator of transcription by binding to DnaA boxes (as before) but the orientation

of the box is not important. What appears important is that at least two DnaA-boxes are present and in the same orientation with respect to each other (W. Messer, personal communication to M. Masters).

In summary DnaA protein has two functions. Its major function is in the initiation of chromosomal DNA replication from *oriC* and other DNA replicon origins, but it also serves as a negative regulator of gene expression, probably by binding to suitably positioned DnaA-boxes.

1.7.2 Suppression of dnaA *mutations*

Many genetic studies on the *dnaA* gene have been reported. It has been found that *dnaA*^{ts} mutants have a remarkably high 'reversion' rate resulting in temperature resistance. Only a small fraction of these revertants result from true reversion, that is back mutations in the *dnaA* gene. Most temperature-resistant colonies have resulted from extragenic suppressors of the temperature-sensitive mutation (Atlung, 1981). At least seven loci have been identified as extragenic suppressors of *dnaA*^{ts} (Atlung, 1981), and four separate attempts to clone the *dnaA* gene have resulted in cloned suppressors (Projan and Wechsler, 1981; Takeda and Hirota, 1982; Jenkins *et al.*, 1986; Fayet *et al.*, 1986). The great number of suppressors seen with *dnaA* mutations suggests that the DnaA protein participates in a large variety of complex cellular processes interacting with several different gene products.

There are at least four general mechanisms by which genetic suppression may occur:

(i) *Interactive suppression*. This form of suppression tends to be very informative with respect to determining functional domains of the mutant proteins in question and for implicating other gene products that are capable of interacting with these proteins. Such suppression, which requires the presence of the mutant polypeptide, does not occur with null-mutants and is often found to be allele specific. For example the suppression of *ssb1* and *ssb113* mutations by *groEL441* and *groEL46* respectively, is thought to represent a real interaction that occurs between the Ssb and GroEL proteins *in vivo*.

(ii) *Increased levels of the mutant polypeptide*. In many cases it has been found that increased expression of a mutant gene under

conditions that would normally be non-permissive can lead to an increase in viability. This is presumably owing to the increased amount of mutant protein compensating for the reduced activity, and could occur by improvement of translational and/or transcriptional efficiencies, or the presence of the mutant genes on multicopy replicons. For example, overexpression of the mutant Ssb1 protein in an *ssb1*^{ts} mutant background leads to high-temperature survival (Chase *et al.*, 1983). In the case of *dnaA* mutants it has been shown that most of the temperature-sensitive alleles can be suppressed in this way (see below).

(iii) *Bypass suppression*. Here the requirement for a particular gene product is eliminated. For example, the partial suppression of *rpoH*-deletion mutants by overexpression of the *groE* genes bypasses the requirement for the σ-32 protein. In the case of DnaA, certain mutations allow initiation of DNA replication from origins other than *oriC* by mechanisms that do not require DnaA. Secondary origins are activated in RNase H-mutant strains (see below), or by integrating a DnaA-independent (or 'less' dependent) origin into the chromosome. This latter process is known as integrative suppression and several examples exist (Lindhal *et al.*, 1971; Nishimura *et al.*, 1971; Moody and Runge, 1972; Nishimura *et al.*, 1973; Goebel, 1974; Tresquerres *et al.*, 1975; Chesney and Rothman-Scott, 1978; Molin and Nördstrom, 1980).

(iv) *Informational suppressors*. Other than the classic nonsense-suppressing tRNAs, frameshift (Smith, 1979) and missense (Hill, 1975) suppressors exist. It is characteristic of these suppressors that they exhibit allele specificity independent of the location of the mutation within the gene, suppression depending on the type rather than the location of that mutation. In addition, they exhibit suppression of a wide range of otherwise unconnected genes. This form of suppression is usually not very informative with regards to protein–protein interactions, or the functional domains within the mutant protein.

In general, most extragenic suppressors of *dnaA* mutants will be acting by one of the first three mechanisms.

Suppression of dnaA^{ts} *by* dasA, dasB, dasC *and* dasG *genes*. None of these suppressing mutations (Atlung, 1981) has been precisely mapped on the *E. coli* chromosome. *DasA* has been roughly mapped to 80 minutes on the genetic map, and results in a cold-sensitive phenotype with an increased DNA/protein ratio, and so may be allelic with the *dnaAcos* suppressor isolated by Kellenberger-Gujer *et al.* (1978), which results in increased DnaA protein levels or activity (see below). *DasB* maps close to the *gid* gene near *oriC* and exhibits phenotypes similar to those seen with suppression by inactivation of RNase H (see below) and therefore may be functioning in the same way. *DasC* maps close to the *ilv* genes and is possibly allelic with *rep*, a gene encoding one of the DNA helicases (Denhardt and Iwaya, 1972). The degree of allelic specificity seen with this suppressor (Atlung, 1981) strongly suggests that a form of interactive suppression is occurring. *DasG* maps close to the *dnaC–T* gene cluster and may be allelic with one of these genes. DnaC is intimately involved with DnaA in the early initiation stages of chromosomal replication, and DnaT is a constituent of the primosome, so suppression by one of these genes seems possible. The *dasE* mutation maps at one minute on the genetic map and was originally isolated along with *dasG*. It is unclear whether it is a suppressor in its own right or merely a secondary mutation allowing more efficient suppression by *dasG*. Little is known about the nature of the *dasE* and *dasG* mutations.

Suppression of dnaA *by RNase H mutants*. It has been shown that secondary mutations in the *rnh* gene encoding RNase H can suppress <u>all</u> temperature-sensitive mutations in the *dnaA* gene (including *dnaA*-null strains) (Horiuchi *et al.*, 1984; Lindahl and Lindahl, 1984; Ogawa *et al.*, 1984; Torrey *et al.*, 1984). It has also been shown that these suppressed strains can be deleted for *oriC*, so it seems that the suppression is bypass in nature (Kogoma and Von Meyenberg, 1983). In these cases chromosomal DNA replication initiates from secondary origins. RNase H degrades the RNA moiety in RNA–DNA hybrid duplexes, and so it appears that such structures are not destroyed at secondary origins when RNase H is inactivated and thus chromosomal replication can occur (De Massey *et al.*, 1984). However, both DnaA and RNase H are components of *in vitro* replication systems and so it

is possible that the proteins do interact in some way. For example, DnaA may protect the DNA-bound RNA primers from RNase H during initiation (Ogawa *et al.*, 1985).

Suppression by mutations in the rpoB *gene.* All *dnaA*^{ts} alleles can be suppressed by secondary mutations within the *rpoB* gene, which encodes the RNA polymerase β-subunit, but allele specificity is observed, i.e. a specific *rpoB* mutation which suppresses one *dnaA*^{ts} allele will not suppress a different *dnaA*^{ts} allele (Atlung, 1981; Atlung, 1984; Schaus *et al.*, 1981a; Hansen *et al.*, 1984a). In addition, one amber mutation can be suppressed by an *rpoB* mutation (Schaus *et al.*, 1981a) suggesting that the mode of suppression may be informational or bypass in nature. However, other amber mutants were not suppressed suggesting that at least a partially functional DnaA protein is required. This was confirmed when it was shown that the suppressed amber mutation was mapped to the distal end of the gene leaving most of the protein intact (Schaus *et al.*, 1981b). It could be argued that the mutations in *rpoB* lead to a form of RNA polymerase that increases transcription of the *dnaA* gene and so the mutation is suppressed by overexpression of the mutant form of the protein. Work by Hansen *et al.* (1992) has shown that almost all the *dnaA*^{ts} mutants can be suppressed by overexpression, of the DnaA mutant protein. One allele however, *dnaA601*, is poorly suppressed by overexpression but can be suppressed by a mutation in *rpoB*; this argues against the possibility that the *rpoB* mutations suppress by increasing expression of the *dnaA* genes. Also, if this were the mechanism then the allele specificity seen between *dnaA* and suppressing mutations in *rpoB* would not be expected. It seems that interactive suppression is more likely in these cases especially since DnaA and RNA polymerase are both known to act at an early stage in DNA replication from *oriC* (Fuller and Kornberg, 1983).

Suppression by mutations in the topA *gene.* The temperature-sensitive *dnaA46* mutation can be suppressed by a secondary mutation in the *topA* gene, which encodes topoisomerase I (Louarn *et al.*, 1984). This suppression requires the presence of the mutated DnaA protein. It has been suggested that the suppression could be caused by

increased transcription of the *dnaA* gene owing to alteration of the DNA's superhelical density (Pruss *et al.*, 1982; Dinardo *et al.*, 1982), or that initiation at *oriC* requires less DnaA protein in the absence of topoisomerase I.

Cloned suppressors of dnaA — groE *genes.* There have been several separate reports concerning cloned suppressors of *dnaA*^{ts} mutations (Projan and Wechsler, 1981; Takeda and Hirota, 1982; Jenkins *et al.*, 1986; Fayet *et al.*, 1986). Three of these have been characterized; all are identical (Takeda and Hirota, 1982; Jenkins *et al.*, 1986; Fayet *et al.*, 1986). The others have not been mapped precisely, but have been shown not to suppress amber mutations and so are not thought to be bypass suppressors, nor to be allelic with *dnaB*, *dnaC*, *dnaE*, *dnaZ* or *rpoB*. However, their identity remains obscure. The cloned suppressor, which has been characterized, carries the *groE* genes. Temperature-sensitive *groE* mutants are deficient in chromosomal DNA replication and RNA synthesis at non-permissive temperatures (Wada and Itikawa, 1984). Closer analysis has revealed that in both *groES* and *groEL* mutants DNA synthesis stops very quickly after the shift to restrictive temperatures. This observation implies that the GroE proteins could be involved generally in DNA replication, and not just in the initiation of chromosomal replication, but the effects seen with DnaA and the GroE proteins are a useful starting point for studying the potential roles of the GroE proteins in DNA replication.

Suppression of *dnaA*^{ts} by *groE* has several interesting characteristics. Both GroES and GroEL are required for suppression (Fayet *et al.*, 1986). Only some of the *dnaA*^{ts} mutant alleles are suppressed by *groE*, and amber mutants are not suppressed (Jenkins *et al.*, 1986). The suppression requires overexpression of the *groE* genes, which is usually achieved by their presence on multicopy plasmids, although λ phages carrying *groE* genes can act as suppressors upon multiple lysogeny. Most of the suppressed alleles exhibit a cold-sensitive phenotype with growth at 30°C severely impaired or even absent. There have been other reports of *dnaA*^{ts} suppressors that also possess this cold-sensitive phenotype. The *dnaAcos* allele is a cold-sensitive, temperature-resistant intragenic suppressor of *dnaA46* (Kellenberg-Gujer *et al.*, 1978). Sequence data

have shown that the *dnaAcos* gene contains three point mutations compared to *dnaA*$^+$ and that *dnaA46* has two (Braun *et al.*, 1985; Hansen *et al.*, 1992). At low temperatures (30°C) strains carrying *dnaAcos* exhibit an increased initiation frequency for chromosome replication, suggesting that this may be the cause of cold sensitivity. Overproduction of DnaA46 protein itself does not cause cold sensitivity and can suppress the *dnaA46* phenotype. However, strains carrying *dnaA*$^+$ on the chromosome and *dnaA46* on a multicopy plasmid do show a degree of cold sensitivity (Hansen *et al.*, 1992), and some temperature-resistant merodiploid combinations of the wild-type allele and temperature-sensitive alleles are very cold sensitive (Hansen *et al.*, 1984a). It should be remembered that DnaA protein synthesis is autoregulated, and since DnaA46 protein is at least partially active at temperatures non-permissive for growth (as shown by its self-suppressibility) it could be that it is the autoregulatory capacity of the DnaA46 protein that is lacking, leading to temperature sensitivity. The *dnaAcos* mutation could be interfering with regulation of *dnaA46* in a way which increases high-temperature survival, but has an associated negative effect at low temperatures. The phenotypic parallels between the suppression of *dnaA*^{ts} by *dnaAcos* and overexpression of *groE* are striking. A report by Katayama and Nagata (1991) showed that the cold sensitivity seen when *groE* is overexpressed in *dnaA46* strains is also caused by over initiation of chromosomal replication. They also showed that the effect was not dependent on new protein synthesis, suggesting that the GroE proteins are increasing the capacity of the DnaA46 protein to initiate; they also found that the levels of DnaA46 protein in suppressed and non-suppressed cells were similar, implying that regulation is not involved. Overinitiation in this system was seen to occur repeatedly over a four hour period without any additional protein synthesis, whereas in the case of *dnaAcos* the burst of overinitiation only lasts for one hour after a temperature shift down. This difference may indicate that in the *dnaA46* strain suppressed by overexpression of *groE* the DnaA protein is maintained in an initiation-competent conformation for a much longer period than in *dnaAcos* strains. Very interestingly it was shown that overexpression of the GroE proteins in a *dnaA46*, *cya283* (required for cAMP synthesis) strain allows temperature-resistant growth, but does

not cause cold sensitivity at 30°C in the absence of cAMP. When cAMP is added to the growth medium, however, the cold-sensitive phenotype returns. Cyclic AMP is believed to modulate the availability of replication-competent DnaA molecules by recycling the inactive ADP-bound form to the active ATP-bound form (Hughes *et al.*, 1988). It is tempting to suggest that when GroE proteins are overexpressed in *dnaA46* strains at low temperatures too much of the ATP-bound DnaA protein is generated and thus cold sensitivity ensues. A further clue comes from the nature of the mutations in the *dnaA* temperature-sensitive alleles that are suppressed by overexpression of the GroE proteins. Of the eight different alleles available, five are suppressed. Of these five, four have mutations at position 184 in the protein (as well as a secondary mutation elsewhere) which is believed to be an important amino acid in the ATP-binding domain (Hansen *et al.*, 1992). The *dnaA46* allele carries this mutation and purified DnaA46 protein binds ATP with a much reduced efficiency compared to wild-type protein *in vitro* and is inactive for *in vitro* chromosomal replication initiation with purified enzymes (Hwang and Kaguni, 1988a, b). Maybe the GroE proteins can somehow improve the ATP-binding capacity of DnaA46. One effect that tallies exactly between those alleles that are suppressed by GroE and those that are not is the ability of the mutant proteins to regain function after return to permissive temperatures. Hansen *et al.* (1984a) showed that some of the mutant *dnaA* alleles could successfully reinitiate chromosomal replication after a downshift from non-permissive to permissive temperatures without additional protein synthesis, and it is only those alleles that are suppressed by overexpression of the *groE* genes. It seems unlikely that *dnaA*^{ts} suppression by *groE* genes is simply caused by more of the mutant protein being made competent for replication initiation. If this were so then all *dnaA* mutant alleles would be suppressed since they are virtually all self-suppressible when overexpressed, except for *dnaA601* (Hansen *et al.*, 1992). However, *dnaA601* is suppressed by the *groE* genes.

Another interesting observation is that certain *dnaA*^{ts} alleles exhibit cold sensitivity when expressed in the same cell as the wild-type DnaA protein (Hansen *et al.*, 1984a). Sequence data again reveal that only those alleles with mutations in the ATP-binding domain are

affected in this way (Hansen *et al.*,1992). The *dnaA167* mutant allele is the only one that is suppressed for high-temperature growth by overexpression of the *groE* genes, which is not cold sensitive in the merodiploid form with wild-type DnaA. This mutation is also not cold sensitive in the presence of large amounts of GroE proteins.

Even with this plethora of information it is far from clear what the mechanism is that allows the GroE proteins to suppress certain *dnaA* temperature-sensitive mutants. There are so many different mechanisms by which *dnaA*^{ts} mutations are suppressed, although it was hoped that the process of *groE* suppression of *dnaA* could be understood. It was this phenomenon that was to be the starting point for my studies.

1.8 The project

The phenomenon of suppression of *dnaA* temperature-sensitive mutants by overexpression of the GroE proteins was first reported by Fayet *et al.* (1986), and from our laboratory by Jenkins *et al.* (1986) and is described in the introduction to *Chapter 3*.

Jenkins work was continued by March (1988). In the course of his investigations a derivative of Jenkins' *groE* carrying plasmid pND5 was constructed named pJM32. This plasmid carried the whole *groES* gene and the first 495 amino acids (from a total of 548) of GroEL which was fused to an out of frame region of a tetracycline-resistance gene (this will be explained in more detail in *Chapter 3*). March showed that this construct was present in the cell at a much reduced plasmid copy-number compared to pND5, did not complement *groEL*^{ts} mutants but did suppress *dnaA*^{ts} mutations. March suggested that this was because some sequence had been removed from the GroEL protein which allowed more 'freedom' for the protein to interact with DnaA protein. One region of the removed sequence which seemed interesting was the extreme carboxyl-terminus of the protein. This region comprises a striking tandemly repeated glycine–glycine–methionine motif. Furthermore this sequence looks as if it could be hydrophobic and thus possibly a membrane anchor, the removal of which could possibly have allowed the freedom for the low gene dosage suppression suggested by March. This phenomenon seemed worthy of continued investigation, and it was at this point that I joined the laboratory with the intention of characterizing further the interaction of the GroE and DnaA proteins.

Chapter 2
Materials and Methods

2.1 Bacterial, phage strains and plasmids

Bacterial strains used in this study are listed in Table 2.1.1. Bacteria were either maintained on L-broth plates stored at room temperature, or for longer term storage, in frozen storage buffer at -70°C.

Bacteriophages used in this study are listed in Table 2.1.2. Phage lysates were stored at 4°C as broth suspensions to which a few drops of chloroform had been added to prevent microbial growth. M13 phage lysates were stored without chloroform; M13 being chloroform sensitive.

Plasmids used and constructed in the course of this study are listed in Table 2.1.3.

Growth media and buffers

Growth media and bacterial/phage buffers and other commonly used buffers are listed in Table 2.1.4. L-broth and L-agar were used routinely for all bacterial manipulations, except where stated. For work with phage λ the media were supplemented with 10mM $MgSO_4$ and 0.2% maltose to maximize expression of the λ receptor protein; for phage P1 2.5 mM $CaCl_2$ was added. VB minimal agar, supplemented with appropriate carbon sources, vitamins and amino acids, was used for selection of nutritional markers.

Growth of Bacteria

Bacteria were routinely grown as liquid cultures at 37°C (or 30°C for temperature-sensitive strains). Usually fresh overnight cultures that had been inoculated from a single colony were diluted back the following day and grown as required.

Bacterial strains, phages and plasmids

Table 2.1.1 Bacterial strains

Strain	Genotype	Source/Reference
AG31	F-, *groES131*^{ts}	C Georgopolous
AG40	F-, *groEL100*^{ts}	C Georgopolous
C600	F-, *thi1*, *leuB6*, *thr1*, *lacY1*, *hsdR*, *tonA21*, *supE44*	Young and Davis (1983)
CB71-18	F'[*proAB*$^{+}$,*lacIq*, *lacZΔM15*] *thi1*, *supE*, Δ(*lac-proAB*)	C Boyd
CG714	F-, *groEL140*^{ts}	C Georgopolous
DH1	F-, *thi1*, *relA1*, *recA1*, *endA1*, λ-, *spoT1*, *hsdR17*, *gyrA96*, *supE44*	D Hannahan
DL51	F-, *recB21*, *recC22*, *sbcB15*, *sbcC*	D Leach
DL307	F-, *recD*	D Leach
HX24	F-, *groES30*^{ts}	R Hendrix
HX39	F-, *groES619*^{ts}	R Hendrix
HX369	F-, *groEL44*^{ts}	R Hendrix
JC10-240	Hfr : P045 *lysA* > *serA* *thi1*, *ilv318*, *thr300*, *srlC300*::Tn *10* *recA56*, *relA1*, *rpsE300*, Spc^{R}, λ-	AJ Clark
MM7	F-, *argG6*, *hisG1*, *ilv*, *leuB6*, *metB1*, *pyrE*, *gal6*, *lacY1*, *malA;* λ^{R} *xyl7*, *supE44*, *uhp*, *fhuA2*, *gyrA;* Nal^{R}, *rpsL104*, *tsx*, λ-	Laboratory stocks
MM18	F-, *argG6*, *asnA31*, *asnB32*, *hisG1*, *leuB6*, *metB1*, *pyrE*, *gal6*, *lac*, *xyl7*, *supE44*, *uhp*, *bgl*$^{+}$, *fhuA2*, *gyrA;* Nal^{R},*rpsL*, *tsx*, λ-	Laboratory stocks
MM38	As MM18, except *asn*$^{+}$	Laboratory stocks
MM38pcn	As MM38, except *pcnB*Δ::Kan^{R}	Laboratory stocks
MM181	As MM18, except *asnA*$^{+}$, *dnaA167*^{ts}	J March (1988)
MM182	As MM18, except *asnA*$^{+}$, *dnaA5*^{ts}	J March
MM183	As MM18, except *asnA*$^{+}$, *dnaA204*^{ts}	J March
MM184	As MM18, except *asnA*$^{+}$, *dnaA508*^{ts}	J March
MM185	As MM18, except *asnA*$^{+}$, *dnaA46*^{ts}	J March
MM186	As MM18, except *asnA*$^{+}$, *dnaA203*^{ts}	J March

Strain	Genotype	Source/Reference
MM187	As MM18, except *asnA*$^{+}$, *dnaA602*^{ts}	J March
MM188	As MM18, except *asnA*$^{+}$, *dnaA601*^{ts}	J March
MM189	As MM18, except *asnA*$^{+}$, *dnaA604*^{ts}	J March
MM19	F-, *argG6*, *asnA31*, *asnB32*, *hisG1*, *leuB6*, *metB1*, *pyrE*, *gal6*, *lacY1* *xyl7*, *supE44*, λ-, *fhuA2*, *gyrA*; Nal^{R} *rpsL104*, *tsx1*, *uhp*, *dnaA46*^{ts}	Laboratory stocks
MM21	As MM19, except *recA56*, *srlC300*::Tn *10*, Tet^{R}	Laboratory stocks
MM22	As MM21, except Tet^{S}	Laboratory stocks
MM306	F-, *thi1*, *ilv192*, *argH1*, *metB1*, *hisG1*, *xyl7*, *lacY1* or *Z1*, *tnaA1*, λ-, *strA*, *ara13*, *tsx7*, *supE44*, (P1), *tryp* Δ, *uhp*, *pyrE*, *purA*	Laboratory stock
NL1	As PC0698, except *purA*$^{+}$	This work
NL13	As NL1, except *recA56*, *srlC300*::Tn *10*, Tet^{R}	This work
NL19	As NM306, except *ilv*$^{+}$, *dnaA46*^{ts}	This work
NL30	As NL1, except *groES30*^{ts}	This work
NL44	As NL1, except *groEL44*^{ts}	This work
NL100	As NL1, except *groEL100*^{ts}	This work
NL131	As NL1, except *groES131*^{ts}	This work
NL191	As NL19, except *dnaA46*^{ts}- Tn*7* , Tmp^{R}	This work
NL192	As NM306, except *purA*$^{+}$	This work
NL192Ω	As NL192, except *groESL*Δ::Ω, Str^{R}/Spc^{R} Requires *groE* complementation in trans	This work
NL193Ω	As NL192Ω, except *dnaA*$^{+}$ Tn*7*, Tmp^{R} Requires *groE* complementation in trans	This work
NL194Ω	As NL193Ω, except *dnaA46*^{ts} Tn*7*, Tmp^{R} Requires *groE* complementation in trans	This work
NL302	As NL30, except *recA56*, *srlC300*::Tn *10*, Tet^{R}	This work
NL441	As NL44, except *recA56*, *srlC300*::Tn *10*, Tet^{R}	This work
NL619	As NL1, except *groES619*^{ts}	This work
NM306	As MM306, except *met*$^{+}$	This work
OF216	F-, *thi1*, *leu*, *thyA*, *deoB* or *C*, *supE42*, *groES3*::Ω (λ117, *groE*$^{+}$)	Fayet *et al.* (1989)
OV32	F-, *htpR165*$^{ts(amber)}$	A Kumar

Strain	Genotype	Source/Reference
PC0698	F-, *thr20, leu32, proA35, codA2, lacY1, tsx70, gal6*, λ^s, λ^-, *rpsL125, xyl7, mtl2, pyr63, purA45*	Laboratory stocks
RM121	F-, *ssb1*^{ts}	Ruben *et al.*(1988)
RM139	F-, *ssb113*^{ts}	Ruben *et al.*(1988)
TG1	F'[*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZΔM15*] *thi*1, *supE*, *hsdΔ5*, Δ(*lac-proAB*)	Gibson 1984
TP91	F-, *dnaA*$^+$ Tn*7*, Tmp^R	T Paterson
W3110	F-, INV: *rrnD-rrnE*, *sup*^{0}, λ^s, λ^-	Laboratory stock

Table 2.1.2 Bacteriophages

Bacteriophage	Description	Source/Reference
P1	Wild-type transducing phage	Laboratory stock
P1*vir*	Virulent transducing phage	Laboratory stock
T4	Wild-type	Laboratory stock
T5	Wild-type	Laboratory stock
λ wild-type	Wild-type immunity	Laboratory stock
λ*vir*	Virulent	Laboratory stock
λ*sidA*	*lac5*, *att*$^+$, *imm21*, *cI*$^+$, *ninR5*, 8.1kb *groE*$^+$ insert	Jenkins (1985)
λ*sidΩ1*	As λ*sidA*, except *groESL*Δ::Ω, Str^R/Spc^R	This work
M13mp18	M13 based cloning vector	Laboratory stock
M13ESL 8	M13 mp18 + 2.1kb *groE*$^+$ insert	This work

Table 2.1.3 Plasmids

Plasmid	Description	Source/reference
pBR322	Amp^R, Tet^R, pMB1 replicon	Bolivar (1978)
pBR325	Amp^R, Tet^R, Chl^R, pMB1 replicon, (*oriT*$^+$)	Bolivar (1978)
pGroEL	Chl^R, pACYC + 2.1kb *groES*$^-$,*EL*$^+$ insert	Goloubinoff *et al.* (1989b)
pGroESL	As pGroEL, except *groES*$^+$,*EL*$^+$	Goloubinoff *et al.* (1989b)
pGT3270	pJF118EH + 2.1kb *groE*$^+$ insert	This work
pGTHC18	pJF118EH + 5.0kb *groES*$^+$, EL_{tr} insert	This work
pGTIR88	pJF118EH + 5.0kb *groE*$^+$ insert	This work
pHC18	pVH1 + 8.1kb *groES*$^+$,EL_{tr} insert	This work
pHC23	As pHC18, except carries a 200bp *Pvu*II deletion	This work
pHCF3	pML31 + 8.1kb *groES*$^+$,EL_{tr} insert	This work
pHCΩ1	pIR88, except *groE* genes replaced with Ω fragment (Str^R/Spc^R)	This work
pHCΩ2	As pHCΩ1, except *groES*$^+$ i.e. only *groEL* replaced	This work
pHP45Ω	Amp^R, pBR based plasmid carrying 2.0kb Ω fragment (Str^R/Spc^R)	Prentki and Krisch (1984)
pHR277	Kan^R, mini-F based plasmid with unique *Eco*RI and *Hin*dIII cloning sites	H Hara

Plasmid	Description	Source/reference
pHSP60	Amp^R, pBR based plasmid with 5.0kb HSP60$^+$ insert from yeast	Reading *et al.* (1989)
pIR88	pVH1 + 8.1kb *groE*$^+$ insert	IR Oliver
pIR89	pVH1 + *groE*'insert from pJM32	IR Oliver
pIR2010	Amp^R, Tet^S, Chl^S, pBR325 derivative (*oriT*$^+$)	IR Oliver
pIRF1	pML31 + 8.1kb *groE*$^+$ insert	This work
pJF118EH	Amp^R, cloning/expression vector *lacI*^{q}, *tac* promotor. EH refers to the orientation of the polylinker	Furst *et al.* (1986)
pJM32	Amp^R, Tet^S, 8.1kb derivative of pND5 carrying *groES*$^+$ and N'- terminal of *groEL* on a 3.7kb *Eco*RI, *BamH*I fragment	March (1988)
pML31	Kan^R, 16kb mini-F derivative	Lovett and Helinski (1976)
pLT548	pHR277 based plasmid containing 2.1kb *groESL*$^+$ *Eco*RI-*Hin*dIII DNA fragment from M13ESL8. Other pLT plasmids are numbered according to the length in amino acids of the GroEL protein they encode.	This work
pMyco65	Amp^R, pBR based plasmid carrying *groE* genes from *Mycobacterium leprae*	D Young
pND5	Amp^R,Tet^R, Chl^S derivative of pBR325 carrying an 8.1kb *groE*$^+$ insert	Jenkins *et al.* (1986)
pRsbp α	Amp^R, pBR based plasmid carrying the rubisco subunit binding protein α-subunit from wheat	J Ellis
pS4	Amp^R, pBR based plasmid carrying the *groES* gene only	Fayet *et al.* (1989)
pVH1	Kan^R, *lacI*^{q}, *colD* replicon, compatible with pBR based plasmids	M Bagdasarian

Table 2.1.4 Growth Media

L-broth	Difco bacto tryptone	10 g
	Difco bacto yeast extract	5 g
	NaCl	5 g
	pH to 7.2 with NaOH	
	Distilled water to 1 litre	
L- agar	L-broth + 15 g Difco agar per litre	
LB top agar	L-broth + 6.5 g Difco agar per litre	
Nutrient broth (NB)	Oxoid No.2 nutrient broth	25 g
	Distilled water to 1 litre	
	It should be noted that NB has insufficient thymine, this was therefore routinely added at a concentration of 40 $\mu g\ ml^{-1}$	
NB agar	Nutrient Broth + 12.5 g Davis NZ agar	
MacConkey agar	Peptone	20 g
	Bile salts No.3	1.5 g
	NaCl	5 g
	Neutral red	0.03 g
	Crystal violet	0.001 g
	Difco agar	15 g
	Distilled water to 1 litre	
VB minimal media	20x VB salts	50 ml
	20% carbon source	10 ml
	Supplements as required	
	Distilled water to 1 litre	
VB minimal agar	As VB minimal media + 15 g Difco agar per litre	

20x VB salts	$MgSO_4.7H_2O$	4 g
	Citric acid	40 g
	KH_2PO_4	400 g
	$NaNH_4.HPO_4.4H_2O$	70 g
	Distilled water to 1 litre	

Commonly used buffers

Phage buffer	Na_2HPO_4	7 g
	KH_2PO_4	3 g
	NaCl	5 g
	$MgSO_4$ (0.1 M)	10 ml
	$CaCl_2$ (0.1 M)	10 ml
	1% gelatin solution	1 ml
	Distilled water to 1 litre	

Bacterial buffer	KH_2PO_4	3 g
	Na_2HPO_4	7 g
	NaCl	4 g
	$MgSO_4.7H_2O$	2 g
	Distilled water to 1 litre	

TE buffer	10 mM Tris–HCl (pH 8.0)	
	1 mM EDTA (pH 8.0)	

TAE buffer	*Working solution*:	
	40 mM Tris–acetate	
	2 mM EDTA	
	50x Conc. stock solution:	
	Tris base	242 g
	Glacial acteic acid	57.1 ml
	0.5 M EDTA (pH 8.0)	100 ml
	Distilled water to 1 litre	

TBE buffer	*Working solution*:	
	89 mM Tris–borate	
	89mM boric acid	
	5x Conc. stock solution:	
	Tris base	54 g
	Boric acid	27.5 g
	0.05 M EDTA (pH 8.0)	20 ml
	Distilled water to 1 litre	

Minimal medium supplements

Amino acid supplements were stored in stock solutions of pure amino acids at a concentration of between 2–10 mg ml^{-1} depending upon the solubility of the particular amino acid. Sparingly soluble amino acids, such as tyrosine, were dissolved in 0.01M NaOH. The final concentration of the amino acids in the media was usually in the order of 20–100 μg ml^{-1}. If a rich minimal medium was required, vitamin-free casamino acids (CAA) were used. The stock concentration of CAA was 100 mg ml^{-1} and the final concentration in the medium was typically 1–5 mg ml^{-1}. It should be noted that casamino acids lack tryptophan and this should therfore be added to CAA medium if the bacterial strain to be used is auxotrophic for this amino acid.

Purines and pyrimidines were added to minimal media when required. Thymine and uracil were stored at a concentration of 2 mg ml^{-1} in water, and their final concentration in minimal medium was usually 20–40 μg ml^{-1}.

The only vitamin supplement found necessary in the entire course of this work was thiamine hydrochloride (vitamin B1). This was stored as a 1 mg ml^{-1} solution in water and its final concentration in minimal medium was 2 μg ml^{-1}.

Selection of Antibiotic Resistance

The routine concentrations for the antibiotics used in this work are shown in Table 2.5. All antibiotics were used in both complex and minimal media with the exception of trimethoprim, which was only used in minimal medium.

Table 2.5 Antibiotic Solutions

	Abbreviation	Solvent	Conc. of stock solution (mg ml^{-1})	Final conc. in media (μg ml^{-1})
Ampicillin	Amp	H_2O	100	50–100
Chloramphenicol	Chl	Ethanol	20	25
Kanamycin sulphate	Kan	H_2O	25	25–50
Naladixic acid	Nal	0.1 M NaOH	20	20
Spectinomycin dihydrochloride	Spc	H_2O	50	25–50
Streptomycin sulphate	Str	H_2O	100	20
Tetracycline hydrochloride	Tet	50% ethanol	10	10
Trimethoprim	Tmp	Methanol	5	50

2.2 DNA Techniques

2.2.1 Large-scale plasmid preparation

A single colony of the plasmid-carrying bacterial strain was inoculated into 5 ml of L-broth with the appropriate selection and incubated overnight at 37°C with vigorous shaking. One millilitre of this culture was then used to inoculate 500 ml of L-broth, with similar selection, in a 2 litre flask, which was then incubated at 37°C overnight, again with vigorous agitation. The culture was chilled on ice, transferred to two 250 ml centrifuge bottles and centrifuged using a Sorval GSA rotor at 5000 r.p.m. for 10 min at 4°C. The bacterial pellets were then each washed in 100 ml of TE buffer, pooled to give a total volume of 200 ml, and recentrifuged as above. The resultant cell pellet was resuspended in 5 ml of a solution containing 50mM Tris–HCl (pH 8.0), 25% sucrose and transferred to a 50 ml centrifuge tube. One millilitre lysosyme (20 mg ml^{-1}) was added, the solution mixed thoroughly, and incubated on ice for 10min. One millilitre of 0.5M EDTA(pH 8.0) and 0.8 ml of RNase A solution (10 mg ml^{-1}) were added and incubation continued for a further 10 min on ice. Finally 5 ml of a lysis solution containing 100 mM Tris–HCl(pH 8.0), 125 mM EDTA, and 0.2% (w/v) Triton X-100 was added, the solution mixed thoroughly and incubated on ice for another 10 min. The resulting suspension was then centrifuged using a Sorval SS-34 rotor at 15,000 r.p.m. for 20 min at 4°C. The plasmid-containing supernatant could now be subjected to isopycnic gradient ultracentrifugation to separate plasmid and chromosomal DNA.

CsCl (17.1 g) was dissolved in the supernatant in a 25 ml measuring cylinder. Ethidium bromide (0.342 ml) solution (10 mg ml^{-1}) was added and the total volume made up to 23 ml with TE. This gave a CsCl density of 1.55 g ml^{-1} and an ethidium bromide concentration of 200 μg ml^{-1}. The solution was then transferred to two 11.5 ml Sorval Ti-50 crimp-seal centrifuge tubes, balanced to within 0.05 g and then centrifuged in a Sorval 50-B or 55-B ultracentrifuge at 38,000 r.p.m. for 60 h at 20°C in a Ti-50 rotor. At the end of the run the tubes were carefully removed from the rotor and the DNA bands could be visualized using a UV lamp. The lower (denser) plasmid bands were removed from the tubes using a syringe

fitted with a wide-bore needle. The two samples were then pooled and the ethidium bromide extracted at least five times with isobutanol (isobutanol over CsCl-saturated TE). The sample was then dialysed against several changes of TE (1:2500) at 4°C over a period of 48 h to remove the CsCl. The plasmid DNA could then be recovered from solution by precipitation.

2.2.2 DNA precipitation

DNA was precipitated from aqueous solution by;

(i) Adding 1/10 volume of 3M sodium acetate (pH5) and 3 volumes of absolute ethanol, mixing thoroughly and leaving on ice for a minimum of 10 min. This was then centrifuged in a microfuge at 15,000 r.p.m. for at least 15 min. The supernatant was discarded, and the pellet washed in 70% ethanol by vortexing. This was recentrifuged as above for 10 min, the supernatant again discarded, and the pellet was dried under vacuum. The dried DNA pellet could then be resuspended in a suitable volume of TE buffer (with added RNase (20 $\mu g\ ml^{-1}$) if required).

(ii) Instead of 3 volumes of absolute ethanol, 1 volume of isopropanol could be used. This had the advantage of keeping the total volume smaller and was therefore the preferred method. After isopropanol precipitation and centrifugation, the pellet was washed with 70% ethanol as above.

2.2.3 Determination of DNA concentrations

DNA concentrations were determined by measuring the absorption of diluted solutions at 260 nm. For double-stranded DNA, an OD_{260} value of 1.0 represents a DNA concentration of 50 $\mu g\ ml^{-1}$, and for single-stranded DNA a similar value represents a DNA concentration of 40 $\mu g\ ml^{-1}$.

DNA purity can be determined by measuring absorption at 260 and 280 nm. Protein-free double-stranded DNA should give a 260/280 ratio close to 1.8, and single-stranded DNA should give a ratio nearer 2.0.

2.2.4 Small-scale plasmid preparation

Routine preparations of plasmid DNA were performed using a modification of the alkaline lysis method of Birnboim and Doly (1979). Five milliltres of L-broth (plus suitable antibiotic selection) was inoculated with a single colony of the plasmid-bearing strain, and incubated overnight with continuous shaking at the appropriate temperature (typically 37°C). The culture was then centrifuged at 3600 g for 10 min in a bench-top centrifuge. The supernatant was discarded, and the bacterial pellet resuspended in 0.1 ml of buffer containing 1% glucose, 10mM EDTA, and 25mM Tris–HCl (pH 8.0). To this cell suspension 0.2 ml of 0.2M NaOH/1%SDS was added, mixed by gentle inversion of the tube and incubated on ice for 5 min. One hundred microlitres of 3 M Na acetate (pH 5.0) was then added, the solution mixed vigorously by extensive vortexing, and left on ice for a further 5 min. The mixture was then centrifuged in a microfuge for 10 min in order to pellet the precipitated chromosomal DNA and insoluble cellular debris. The resulting supernatant (~0.5 ml) was transferred to a fresh Eppendorf tube and 0.5 ml of phenol/chloroform (phenol saturated with TE (pH 8.0) plus an equal volume of chloroform) was added, mixed by vortexing and centrifuged in a microfuge for 2 min. The upper aqueous phase was transferred to a fresh tube and the plasmid DNA could then be recovered from solution by ethanol or isopropanol precipitation. In this case no extra salt needed to be added in order for precipitation to occur. Typically the final pellet of nucleic acid is resuspended in TE buffer containing RNase A (20 μg ml^{-1}). It was usually observed that 5 ml of overnight culture yielded approximately 3–5 μg of plasmid DNA.

2.2.5 Restriction of DNA

Endonuclease cutting of DNA was typically performed in volumes of between 20 and 100 μl. These contained the requisite amount of DNA (usually 1–10 μg) and the appropriate Boehringer Mannheim restriction buffer at 1x concentration. The restriction enzyme was usually present in a two- to fivefold excess, i.e. 2–5 units per microgram of DNA. The digests were made up to their final volume using distilled water. The complete restriction digests were incubated at the recommended temperature (usually 37°C) for 1–3 h. The

products of the reaction were either directly analysed by agarose gel electrophoresis, or phenol extracted, ethanol precipitated and dissolved in a suitable volume of TE buffer for further manipulations.

Partial digestion of DNA. For partial digestion of DNA, ten two-fold serial dilutions of restriction enzyme were added to fixed amounts of DNA, with 0.5 units of enzyme μg^{-1} DNA representing the highest enzyme:DNA ratio. The digests were incubated at the appropriate temperature for 1 h and terminated by addition of tracking dye (see *2.2.8*). The products of the reactions could then be analysed by agarose gel electrophoresis.

2.2.6 Ligation of DNA

Ligations of DNA were typically performed in a final volume of 10 µl. These contained between 0.5–1 µg total DNA with insert DNA in a 2- to 20-fold molar excess over the vector DNA, 1x Boehringer Mannheim ligation buffer and T4 DNA ligase. Ligase (0.2 units) was used for the ligation of cohesive DNA termini, and 1 unit of the enzyme for the ligation of blunt-ended molecules. The reactions were incubated for at least 12 h at 16°C. Between 5 and 10 µl of the reaction mixture was then used to transform competent cells of an appropriate strain of *E. coli.*

2.2.7 'Filling in' of recessed 3' termini

Klenow enzyme was used to fill-in the recessed 3' termini generated by various restriction enzymes to give blunt-ended DNA molecules. Reactions were performed in a final volume of 20 µl containing 1µg DNA, 1x Klenow buffer, all four dNTPs each at a concentration of 20 µM and 2 units of Klenow enzyme. The reactions were incubated at 16°C for 45 min. The reactions were stopped and the unincorporated nucleotides removed by increasing the reaction volume to 200 µl with TE, phenol extracting and ethanol precipitating the DNA.

2.2.8 Agarose gel electrophoresis

Agarose gel electrophoretic analysis of DNA was always performed using TAE buffer. The gels were made-up by melting the appropriate amount of agarose (usually between 0.8 and 1.5%) in 1x TAE buffer

using a microwave oven. Gels were cast in 11 x 14 cm Pharmacia gel trays, and once set the DNA samples containing 1x tracking dye (6x tracking dye is 0.25% bromophenol blue, 0.25% xylene cyanol and 40% (w/v) sucrose in H_20) were loaded into the wells at one end of the tray. Gels were run in Pharmacia gel electrophoresis tanks with the gels only just immersed in 1x TAE buffer. Electrophoresis was usually performed overnight at a constant current of 25 mA. After completion of electrophoresis, gels were stained in water containing 2 μg ml^{-1} ethidium bromide for about 1 h with constant shaking, and subsequently destained in fresh water for 30 min. The gels could then be photographed using Polaroid film and UV transillumination.

2.2.9 Isolation of DNA from agarose gel slices

To isolate DNA from agarose gels the Geneclean method was employed. Geneclean is a product of Bio101 and utilizes a silica matrix (glassmilk) which binds DNA in high-salt but not in low-salt solutions. The appropriate DNA band is located in an ethidium bromide-stained gel under UV transillumination and cut out using a clean razor blade in as small a volume of agarose as possible. Gel slices were transferred to Eppendorf tubes, the weight of the slice determined and 3 volumes of saturated sodium iodide solution added. These were incubated at 50°C until the gel slice had dissolved. Five microlitres of glassmilk was added, the suspension mixed well and put on ice for 5 min. Tubes were briefly spun in a microfuge and pellets washed three times in 0.25–0.5 ml of Geneclean New-wash solution (an alcohol-based washing solution supplied with the kit), centrifuging and resuspending the pellets each time. After the final wash, all traces of the wash solution were removed using a Pasteur pipette and the pellets were then suspended in 5 μl TE buffer. These were incubated at 50°C for 2–3 min, centrifuged for 30 seconds and the DNA-containing supernatant transferred to a fresh tube. A further 5 μl of TE buffer was added to the glassmilk pellets and the procedure was repeated to give a final DNA-containing solution with a volume of 10 μl. This DNA solution could be directly used for further manipulations.

2.2.10 Labelling DNA fragments by nick-translation

The labelling of DNA fragments was performed using the nick-translation method of Rigby *et al.* (1977) using a convenient kit (Boehringer Mannheim). The total volumes of the nick-translation reactions were 20 µl each. These contained up to 2 µg of the DNA to be labelled, 20 µM dA, dC, dGTPs, 1x nick-translation buffer, and the non-radioactive label biotin-11-dUTP (Sigma) at a concentration of 20 µM. To these 1 µl of DNase I/DNA-polymerase I enzyme mix was added, mixed well and incubated at 16°C for 1 h. The reaction was stopped and the unincorporated nucleotides removed by phenol extraction and ethanol precipitation. The resulting DNA pellets were dissolved in 50 µl TE. DNA labelled with biotin in this way is stable for months at –20°C.

2.2.11 Preparation of chromosomal DNA

A single colony of the appropriate strain of *E. coli* was used to inoculate 5 ml of L-broth, which was incubated overnight at 37°C, (or 30°C for temperature-sensitive strains) with vigorous shaking. One millilitre of this culture was used to inoculate 100 ml of L-broth, which was again incubated at a suitable temperature overnight with constant agitation. The culture was then chilled on ice and transferred to a 250 ml centrifuge bottle and centrifuged at 5000 r.p.m. for 15 min at 4°C using a Sorval GSA rotor. The supernatant was removed and the bacterial pellet was resuspended in 20 ml of STE, which is TE buffer with 10 mM sodium chloride. One millilitre of 10% SDS solution and 1 ml of proteinase K solution (4 mg ml^{-1}) were added, mixed gently and incubated at 50°C for 6 h without shaking. To this solution an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, the suspension mixed gently by inversion and allowed to stand at room temperature for 10 min. This was centrifuged in a benchtop centrifuge for 15 min to separate the aqueous and phenolic phases. The upper aqueous phase was then carefully removed avoiding the protein interface. The nucleic acids in this phase were precipitated by adjusting the sample to 0.2 M sodium acetate (pH 5.5) and gently layering 2 volumes of ice-cold ethanol on top. The DNA was collected at the aqueous–ethanol interface by spooling it out with a glass rod.

The spooled DNA was washed in 70% ethanol, dried briefly in air and dissolved overnight in 10 ml of TE at room temperature. To this solution 0.1 ml of RNase A (10 mg ml^{-1}) was added and the mixture incubated at 37°C for 1 h. Five hundred microlitres of a 10% SDS solution and 250 µl of proteinase K solution (4 mg ml^{-1}) were now added and the mixture incubated at 50°C for 1 h. The sample was extracted with phenol:chloroform:isoamyl alcohol and the DNA precipitated by spooling as above. After washing in 70% ethanol the DNA was air dried and solubilized in 1 ml of TE. This solubilization took between 1 and 3 days. The yield of DNA was determined by UV spectrophotometry as described previously; from 100 ml of culture about 500 µg of chromosomal DNA was typically obtained.

2.2.12 Preparation of bacteriophage M13 DNA

In all preparations of M13 DNA the *E. coli* strain TG1 was used. A 5 ml overnight culture of TG1 was prepared in the usual way. This culture was used to set up a culture infected with bacteriophage M13. Fifty microlitres of the overnight culture was used to inoculate 2 ml of L-broth. To this either 100 µl of an M13 phage suspension, (about 1/10 of a single plaque), or an entire M13 plaque from an agar plate was added. This culture was incubated at 37°C with vigorous shaking for about 5 h. One-and-a-half millilitres of this culture was transferred to an Eppendorf tube and centrifuged in a microfuge for 5 min. The resulting bacterial pellet could be used to prepare the double-stranded replicative form of M13 DNA, and the supernatant used to prepare single-stranded M13 DNA (or as a fresh bacteriophage suspension).

Preparation of double-stranded M13 DNA. The bacterial pellet was washed once in bacterial buffer and the double-stranded DNA isolated in essentially the same way as was described for the small-scale isolation of plasmid DNA.

Preparation of single-stranded M13 DNA. Between 1.2–1.3 ml of the bacteriophage suspension was transferred to an Eppendorf tube and 200 µl of a solution containing 20% polyethylene glycol (PEG8000) in 2.5 M sodium chloride was added, the solution mixed thoroughly and

allowed to stand at room temperature for 15 min. The precipitated bacteriophage particles were recovered by centrifuging in a microfuge for 5 min and the supernatant was removed carefully leaving the pellet as dry as possible. The bacteriophage pellet was resuspended in 100 µl TE with vigorous vortexing. Fifty microlitres of phenol (equilibrated with Tris–HCl (pH 8.0)) was added and the suspension mixed thoroughly by vortexing for 1 min. This was then centrifuged for 2 min and the upper aqueous layer carefully removed and placed in a fresh tube. The volume of the sample was adjusted to 0.5 ml and the single-stranded DNA recovered by phenol–chloroform extraction and ethanol precipitation as has been described earlier. An optional step was to chloroform extract the sample after the phenol–chloroform extraction. Using this method the yield of single-stranded DNA tended to be approximately 5–10 µg DNA per millilitre of infected culture and was of sufficient quality for both DNA sequencing and site-directed mutagenesis reactions.

2.2.13 Southern blotting procedures

Transfer of DNA from agarose gels to nitrocellulose filters. Chromosomal DNA was digested with the desired restriction enzymes, electrophoresed through agarose, stained and photographed as has been described above. The DNA fragments were depurinated by soaking the gel in 2 volumes of 0.25 M HCl with gentle agitation for 15 min. This was repeated with fresh 0.25 M HCl for a further 15 min. The gel was then rinsed with distilled water and the DNA denatured by soaking in 2 volumes of 0.5 M NaOH/1.5 M NaCl with gentle agitation for 15 min. This was repeated with fresh denaturation solution for a further 15 min. Preceding transfer of the DNA to nitrocellulose, the gel was soaked in 2 volumes of 1.0 M ammonium acetate/0.02 M NaOH with agitation for 30 min. This treatment was repeated for a further 30 min with fresh solution. Ammonium acetate is used for transfer because of the high binding capacity of nitrocellulose for single-stranded DNA in this buffer (Smith and Summers, 1980).

A sheet of nitrocellulose saturated with 1.0 M ammonium acetate/0.02 M NaOH was placed on top of three sheets of blotting paper (saturated with the same buffer) on a clean glass plate. The

nitrocellulose filter and the blotting paper were cut to the same size as the gel. The pretreated gel was placed on top of the nitrocellulose, taking care to exclude any air bubbles; this was followed by a further three sheets of saturated blotting paper. On top of this a 2–4 cm thick wad of dry paper towels was placed, followed by another glass plate. The whole 'sandwich' was then inverted so that the glass plate which formed the base could be removed and another wad of dry paper towels placed on top. The glass plate was replaced and the whole structure was weighted to provide even pressure and maintain good contact between the gel, nitrocellulose, and paper towels. Transfer was allowed to continue overnight. After transfer was complete the nitrocellulose sheet was removed, rinsed in 6x SSC for 2 min, blotted dry, and baked at 80°C for 2 h to fix the DNA. Nitrocellulose filters could be stored at this stage until required.

Pre-hybridization of nitrocellulose filters. The nitrocellulose filter was uniformly rehydrated in 2x SSC prior to pre-hybridization. The filter was then placed in a polypropylene bag that had been heat-sealed at three sides. Pre-hybridization solution was added to the bag and the final side of the bag heat sealed, avoiding the formation of bubbles. The volume of pre-hybridization solution used was typically 0.1 ml cm^{-2} of filter. The filter was incubated in the pre-hybridization solution for 4 h at 42°C with constant agitation.

Hybridization of nitrocellulose filters. The pre-hybridized nitrocellulose filter was removed from the bag and placed in a clean polypropylene bag sealed at three sides. To this the hybridization solution was added, along with the freshly denatured biotin-labelled probe DNA. The volume of the hybridization solution was 0.1 ml cm^{-2} of filter, and the probe concentration was 100 ng ml^{-1}. The bag was sealed at its fourth side, again avoiding bubbles, and the filter incubated overnight at 42°C with constant shaking.

The post-hybridization washes were performed as follows. The filter was first washed in 250 ml 2x SSC/0.1% SDS for 3 min at room temperature. This was repeated once. The filter was then washed in 250 ml 0.2x SSC/0.1% SDS for 3 min. This was repeated once. Finally the filter was washed in 250 ml 0.16x SSC/0.1% SDS for 15 min at

50°C. Again this was repeated once. The filter was briefly rinsed in 2x SSC at room temperature and was then ready for developing by detecting the presence of the biotinylated probe DNA.

Solutions required for pre-hybridization and hybridization of nitrocellulose filters.

50x Denhardt's solution.
1% ficoll
1% polyvinylpyrrolidone
1% BSA

Pre-hybridization solution.
50% formamide
5x SSC
5x Denhardt's solution
25 mM sodium phosphate, pH6.5
0.5 mg ml^{-1} freshly denatured, sheared herring-sperm DNA

Hybridization solution.
45% formamide
5x SSC
1x Denhardt's solution
20 mM sodium phosphate, pH6.5
5% dextran sulphate
0.2 mg ml^{-1} freshly denatured, sheared herring-sperm DNA
100 ng ml^{-1} freshly denatured, biotin-labelled probe DNA

2.2.14 Detection of biotinylated probes on nitrocellulose

For the detection of biotin-labelled probes, the BRL BluGENE Non-radioactive Nucleic Acid Detection System was used. This system utilizes the fact that the protein streptavidin has a very high affinity for biotin. In the kit, streptavidin is conjugated to alkaline phosphatase, and it is that enzyme which can shown to be present by using a colorimetric assay, which results in a blue deposit on the nitrocellulose filter. The kit was used according to the manufacturer's instructions.

After the post-hybridization washes the filter was soaked in Buffer 1 for 1 min. (Buffer 1 is 0.1 M Tris–HCl (pH 7.5), 0.15 M NaCl). The filter was then incubated in Buffer 1 plus 5% BSA for 1 h at 65°C. This was to block the protein-binding sites on the filter. The BRL streptavidin–alkaline phosphatase conjugate (SA–AP) was diluted just prior to use in a polypropylene tube, using 1μg of stock solution (1mg ml^{-1}) per 1 ml of Buffer 1. Typically 70 μl cm^{-2} of filter was required. The filter was incubated in the dilute SA–AP solution for 10 min with gentle agitation, occasionally pipetting the solution over the filter. The SA–AP solution was decanted and the filter was washed using a 40-fold greater volume of Buffer 1 than was employed for diluting the SA–AP conjugate. This washing of the filter in Buffer 1 was continued for 15 min with gentle agitation and was repeated once with a similar volume of fresh solution. The filter was finally washed once in 200 ml of 0.1 M Tris–HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$ for 10 min. For visualization of the biotin-abelled probe a solution containing the alkaline phosphate substrate complex NBT/BCIP was prepared. The volume of the solution used in this step was 75 μl cm^{-2} of filter. The solution contained 0.33 μl NBT solution plus 0.25 μl BCIP solution per 75 μl of 0.1 M Tris–HCl (pH 9.5), 0.1 M NaCl, 50mM $MgCl_2$ and was added to the filter in a polypropylene bag which was then heat-sealed. The development was allowed to continue until a satisfactory amount of colour had appeared. This usually occured after about 5 min. Longer incubation times resulted in an increase in unwanted background signals. The fully developed filter was washed in 20 mM Tris–HCl (pH 7.5), 0.5 mM EDTA to terminate the colour reaction. The filter was then dried and stored ready for photographing as required. Such filters should be stored in the dark. Rehydration of the dried filters results in rejuvenation of the colour.

2.2.15 Site-directed mutagenesis

Introduction. Site-directed mutagenesis of DNA was performed using the Amersham 'Oligonucleotide-directed *in vitro* Mutagenesis System'. This system is based on the method of Taylor *et al.* (1985) and results in very high yield of mutated DNA sequences. The method involves a strand-specific selection step, which eliminates the unwanted non-mutant sequences *in vitro*, generating a pure homoduplex mutant DNA

sequence thus avoiding host-mediated repair systems. The Amersham system also incorporates a nitrocellulose filtration step which removes any contaminating single-stranded template DNA, which could cause high levels of non-mutant background.

In the procedure, a mutagenic oligonucleotide is annealed to a single-stranded M13 template and is extended by Klenow enzyme in the presence of T4 DNA ligase to generate a mutant heteroduplex. Selective removal of the non-mutant strand is made possible by the incorporation of a thionucleotide into the mutant strand during *in vitro* synthesis. This makes the DNA resistant to the restriction enzyme *NciI* in that the enzyme can only nick phosphorothioate-containing DNA on the chemically normal strand. Such nicks are sites for the single-stranded exonuclease III, resulting in the digestion of the non-mutant (non-phosphorothioate) strand of the cloned target sequence. The mutant strand is then used as the template to reconstruct the double-stranded closed-circular molecule, thus creating a homoduplex mutant molecule. This is then used to produce M13 phage plaques on a suitable M13-sensitive strain of *E. coli* which can then be used for verification of the intended mutation. The mutagenesis procedure is summarized below.

Mutagenesis reaction. Single-stranded M13 DNA was prepared as before, and adjusted to give a concentration of 1 $\mu g\ \mu l^{-1}$ with TE buffer. Phosphorylated oligonucleotides were purchased from the Oswell DNA Service at Edinburgh University; concentrations were adjusted to approximately 1.6 $pmol\ \mu l^{-1}$. To anneal the mutant oligonucleotide to the single-stranded template the following was added to an Eppendorf tube on ice:

single-stranded DNA template	5 µl
phosphorylated mutant oligonucleotide	2.5 µl
Buffer 1	3.5 µl
water	6 µl
Total	7 µl

The tube was then placed in a 70°C water bath for 3 min followed by 30 min in a 37°C water bath. The tube was then placed on ice. To

synthesize the mutant strand and ligate the resulting heteroduplex the following was added to the annealing reaction:

annealing reaction	17 µl
$MgCl_2$ solution	5 µl
nucleotide mix 1	19 µl
water	6 µl
Klenow enzyme	6 units
T4 DNA ligase	6 units

The contents of the tube were then mixed well and placed in a 16°C water bath overnight. The next stage was to remove unreacted single-stranded DNA by centrifuging the mixture through a nitrocellulose filter unit. In 500 mM NaCl, nitrocellulose binds single-stranded DNA but not double-stranded DNA. To the above reaction, 170 µl of water and 30 µl of 5M NaCl were added. This was then mixed well and transferred to the top-half of a disposable filter unit supplied in the kit and centrifuged at room temperature in a HB–4 rotor at 1500 r.p.m. for 10 min. one hundred microlitres of 0.5 M NaCl was added to the top-half of the unit and it was respun for a further 10 min. The double-stranded DNA was then precipitated by adding 28 µl of 3 M Na acetate and 700 µl of cold 100% ethanol to the filtrate. This was placed on ice for 15 min and then centrifuged for 15 min. The supernatant was carefully removed and the pellet washed in 1 ml of 70% ethanol. This was respun, the supernatant removed and the pellet dried in a vacuum desiccator. The pellet was then resuspended in 25 µl of Buffer 2, and of this 15 µl were stored at –20°C. To the remaining 10 µl of sample 65 µl of Buffer 3 was added along with 5 units of *Nci*I. This was incubated at 37°C for 90 min in order to nick the DNA. The next stage was to digest away the nicked non-mutant strand with exonuclease III. To the above reaction the following were added on ice:

NaCl (500 mM)	12 µl
Buffer 4	10 µl
exonuclease III (25 units μl^{-1})	2 µl

The tube was then incubated at 37°C for 30 min. Fifty units of exonuclease III will digest about 3000 bases in 30 min, which is ample

since the closest *NciI* site to the cloned insert is only 600 bases away. The tube was then placed in a 70°C water bath for 15 min to destroy the enzymes. The final step was the repolymerization of the gapped DNA.

To the reaction mix above the following was added on ice:

nucleotide mix 2	13 µl
$MgCl_2$ solution	5 µl
DNA polymerase I	3 units
T4 DNA ligase	2 units

This mixture was placed in a 16°C water bath for 3 h. Twenty microlitres of this product was then used to transform an M13-sensitive strain of *E. coli* and the plaques generated were screened for the incorporation of the expected mutation by direct sequencing of the phage DNA. (See *Bacterial Techniques* for the preparation of competent *E. coli* for DNA transformation.)

2.2.16 DNA Sequencing techniques

Introduction. DNA sequencing was performed using the Pharmacia T7 Sequencing Kit. The kit is based on the chain-terminating dideoxynucleotide sequencing method developed by Sanger *et al.* (1977). In the original procedure, primer extension was catalysed by the Klenow fragment of *E. coli* DNA polymerase I. In the kit, however, the Klenow enzyme has been replaced by T7 DNA polymerase, which has the advantage of creating longer chain-terminated fragments with a more even distribution of label between fragments. The major practical difference in using T7 DNA polymerase is that the primer extension reactions are performed in two stages, a labelling reaction and a termination reaction. The two stages are required because the enzyme uses dideoxynucleotides very readily, and therefore in order to allow the synthesis of long chain-terminated fragments, dideoxynucleotides are excluded from the first stage of the reaction, being added for the second. Even so, the time required for the reactions using the T7 enzyme is considerably less than those using Klenow enzyme.

Annealing of primer to single-stranded template. The DNA templates used in the sequencing reactions were all single-stranded M13 DNAs and were purified as mentioned previously. The concentration of the template was adjusted to 1 $\mu g\,\mu l^{-1}$ in TE. In most cases the Universal Primer supplied in the kit was found to be suitable. This primer is 17 bp long and is at a concentration of 0.80 μM. If another oligonucleotide is used it should be adjusted to the same concentration.

The following was added to an Eppendorf tube on ice:

template DNA (1$\mu g/\mu l$)		2 μl
primer (0.80 μM)		2 μl
annealing buffer		2 μl
water		8 μl
	Total	14 μl

The contents of the tube were mixed well and incubated at 60°C for 10 min. The tube was then left at room temperature for at least 10 min; if the rest of the sequencing reaction was to be performed at a later time then the tube could be stored at –20°C until required.

Sequencing reaction. For each template to be sequenced, four Eppendorf tubes or wells of a microtitre plate were labelled 'A', 'C', 'G' and 'T' respectively and 2.5 μl of the corresponding dideoxynucleotide mix added to each tube or well. To the tube containing the annealed template and primer the labelling mix, (dCTP, dGTP and dTTP in solution), T7 DNA polymerase and labelled dATP were added as follows:

annealed Template and Primer	14 μl
labelling mix	3 μl
[α-^{35}S] dATPαS	1 μl (=10 μCi)
diluted T7 DNA polymerase (1.5 units μl^{-1})	2 μl

This labelling reaction was incubated at room temperature for 5 min. While this was proceeding the previously dispensed sequencing mixes were incubated at 37°C for one minute in a water bath. After the 5 minute incubation of the labelling reaction, 4.5 μl was added to each

of the prewarmed sequencing mixes and returned to the water bath for a further 5 min to allow chain-termination to occur. Finally, 5 µl of Stop Solution was added to each reaction, which could then be stored at –20°C until required for electrophoresis. When the samples were needed for loading onto the sequencing gel they were heated to 80°C for 2 min to denature the DNA. Immediately after this incubation 1.5 to 2.5 µl of each sample was loaded onto the gel.

DNA sequencing gel electrophoresis. DNA sequencing was performed using a 30 x 40 cm BRL sequencing apparatus. The glass sequencing gel plates were thoroughly cleaned with ethanol and chloroform, assembled using 0.2 mm spacers and taped together carefully to minimize the possibility of leakage.

The gel was prepared by adding together the following:

acrylamide (filtered, 40% w/v)	15 ml
urea	43 g
water	35 ml
10x TBE	10 ml

This was allowed to dissolve with the aid of magnetic stirring. Once dissolved, 1 ml of a 10% ammonium persulphate solution was added followed by 35 µl of TEMED. This was stirred slowly for a few seconds and was then poured between the sequencing plates. The flat edge of a 60-well shark-tooth comb was pushed between the plates to layer the top of the gel. Clingfilm was wrapped round the exposed areas of the gel and each edge of the gel was clamped with bulldog clips. The gel was then set aside for at least 10 min to allow polymerization. Once set, the bulldog clips, tape and comb were removed and distilled water was squirted along the top of the gel. The shark-tooth comb was then replaced with the points downwards just touching the surface of the gel. The gel was then clamped into the sequencing apparatus and 1x TBE solution poured into the top and bottom reservoirs. The gel was then pre-run at about 66 W (~1500 V) for 1 h. After this the gel was ready to be loaded with the sequencing reactions. The samples were loaded in the order A, C, G and T immediately after denaturing the DNA (see above). The gel was then electrophoresed at 66 W until the

blue dye-front ran off the end of the gel. Once electrophoresis was complete the glass plates were removed from the apparatus and the top plate very carefully removed. The bottom plate (with the gel attached) was placed in a fixing bath containing 10% methanol and 10% acetic acid in water for 20 min. The plate and gel were then removed and two damp sheets of blotting paper placed on top of the gel followed by two sheets of dry blotting paper. Even pressure was applied and the papers were peeled off the glass plate taking the gel with them. The gel and paper sandwich was then dried in a vacuum gel-drier for 1 h at 80°C. When dry the gel was placed in an autoradiography cassette and allowed to develop at room temperature. In most cases a good signal was achieved after 24 h.

2.3 Bacterial techniques

2.3.1 Preparation of competent cells and transformation with plasmid DNA

To prepare competent *E. coli* cells the method of Chung *et al.* (1989) was employed. Five millilitres of L-broth was inoculated with a single colony of the appropriate bacterial strain and incubated overnight with shaking at a suitable temperature. This culture was diluted 1 in 100 into fresh L-broth and grown, with good aeration, to an OD_{600} of between 0.3 and 0.4. The culture was chilled on ice, transferred to a universal bottle and centrifuged at 3600 g for 10–15 min. The supernatant was removed and the bacterial pellet resuspended in 0.1x the original volume of ice-cold TSS buffer. At this point the cells could be frozen at –70°C, or could be used immediately for transformation. Freshly prepared cells always gave the highest transformation efficiency. The plasmid DNA (typically 1–100 ng in <10 µl) was added to 0.1 ml aliquots of the competent cells, mixed gently and stored on ice for 15–30 min. After this time between 0.4 and 0.9 ml of LBG (L-broth + 20 mM glucose) was added and the cells incubated at an appropriate temperature for 1 h to allow expression of plasmid antibiotic-resistance genes. Two-hundred microlitres of this mixture was then spread onto antibiotic-containing plates and incubated until bacterial colonies appeared. Whenever a transformation was performed an aliquot of competent cells lacking plasmid DNA was used as a control.

Cells can be transformed with replicative form M13 DNA using this method. In this case the LBG step is omitted and 0.25 ml of M13-sensitive plating cells added to the transformed cells. The mixture is added to 3 ml of molten L-top agar, mixed gently and poured onto a L-agar plate which, once set, is incubated at 37°C overnight, after which M13 plaques should be clearly visible.

TSS buffer:	Difco Bacto Tryptone	10 g
	Difco Yeast Extract	5 g
	NaCl	10 g
	PEG 3350	100 g
	$MgSO_4$	20 mM
	DMSO	50 ml
	PIPES Buffer pH 6.5	10 mM
	distilled water to 1 litre	

2.3.2 Frozen storage of bacterial strains

It was found that strains of *E. coli* could be conveniently stored at -70°C without suffering a dramatic loss of viability, this included strains harbouring plasmids that might otherwise be lost. A fresh 5 ml overnight culture was prepared with antibiotic selection if required. This was centrifuged at 4000 r.p.m. for 10–15 min, the supernatant discarded and the cells resuspended in 0.1x the original volume of Frozen Storage Buffer. The cells were then left on ice for a couple of hours before storing at –70°C.

Frozen storage buffer:	50% bacterial buffer
	50% glycerol (v/v)

2.3.3 Hfr mating of E. coli

Hfr mating was found to be an ideal way of introducing the *recA56* mutant allele into various strains of *E. coli*. The *recA56* donor strain used was JC-10 240. This strain has its Hfr origin of transfer close to the *recA* gene and also carries a tetracycline-resistant Tn*10* in a neighbouring gene (*srl*) so that female cells receiving DNA from JC-10 240 usually co-inherit *recA56* with tetracycline resistance. A 5 minute mating was found to be sufficient for transfer of both markers.

JC-10 240 was grown up in L-broth without shaking (so as not to damage the F pili) until an OD_{540} of 0.1 was reached. The recipient strain was grown to a similar OD with agitation, concentrated 10x by centrifugation and resuspension in fresh L-broth. One hundred microlitres of each strain were gently mixed together in an Eppendorf tube and incubated at a suitable temperature (typically 37°C) for 5 and 15 min. Eight hundred microlitres of LBG was then added to each

tube followed by vigorous vortexing to disrupt mating pairs and incubated at the same temperature as before for 1 hour to allow expression of the tetracycline-resistance gene. Two hundred microlitre aliquots were then plated out on suitable plates, which selected against both JC-10 240 and the original recipient, but not against conjugates. The plates were incubated at an appropriate temperature until colonies appeared. These were screened for the presence of the *recA56* allele by testing their UV sensitivity.

2.3.4 Testing UV sensitivity of recA *strains*

A single colony of the strain to be tested was streaked across an L-agar plate using a sterile toothpick. In addition, single colonies of a known *recA* strain and a wild-type control were streaked across the plate to act as controls. The plate was covered with a piece of card and exposed to successively longer periods of UV irradiation; commonly 0, 10, 20, 40, 60, 80, 100 and 120 sec under an ultraviolet lamp calibrated at 600 ergs mm^2 sec^{-1}. The plate was then incubated overnight. Growth of streaks was then compared for the different time intervals, and an estimate of UV sensitivity made. *RecA* strains tend to be sensitive to a 10 sec dose of UV.

2.4 Phage techniques

2.4.1 Preparation of λ plate lysates

Cells were grown in L-broth + 20 mM $MgSO_4$ and maltose at 0.2% until mid-log phase. Two hundred microlitre aliquots were then mixed with 10^6 phage, incubated at 37°C for 5 min, and 3 ml of L-top agar containing 20 mM $MgSO_4$ and 0.2% maltose added. This was poured onto a L-agar plate, left to set and incubated at 37°C overnight or until visible lysis occured. Five millilitres of phage buffer was then added to the plate and the layer of top agar scrapped off into a sterile 250 ml beaker. A few drops of chloroform were added and the beaker was incubated at room temperature with gentle swirling for 20 min. The contents of the beaker was poured into a universal bottle and centrifuged at 4500 r.p.m.for 10 min. The supernatant was transferred to a fresh half-ounce bottle and stored over a few drops of chloroform at 4°C.

2.4.2 Preparation and selection of λ lysogens

A lawn of the bacterial strain to be lysogenized was made by mixing 0.2 ml of a mid-log phase L-broth culture and 3 ml of L-top agar with 20 mM $MgSO_4$ and 0.2% maltose added. To this approximately 200 λ phage particles were added, and the mixture poured on a fresh L-agar plate. Once set the plate was incubated overnight at 37°C. This should result in the appearance of isolated λ plaques. The centre of a plaque was then touched with a sterile toothpick and streaked out onto another plate, which again was incubated overnight. The resulting single colonies could be tested for the presence of the λ phage. A lysogenized bacterium will now be resistant to lysis by phages with the same immunity as the one used to lysogenize the strain, but will be sensitive to λ phages that are virulent, or carrying a different immunity. This was initially done as a cross-streak on an L-agar plate, and positives from this test were checked more fully by trying to make isolated plaques in a similar way to that mentioned above. Strains that seemed to be lysogens were then finally tested by examining supernatants from L-broth cultures to see if they were producing the correct phage.

2.4.3 UV induction of λ lysogens

Lysogenic bacteria were grown in L-broth + 20 mM $MgSO_4$ at 37°C with vigorous agitation until an OD_{540} of 0.3 was achieved. The cells were harvested by centrifugation and resuspended in 7 ml of 20 mM $MgSO_4$. This was transferred to a sterile glass petri dish and the cells were exposed to 600 ergs mm^2 sec^{-1} of UV irradiation before being diluted fivefold in fresh L-broth + 20 mM $MgSO_4$. This culture was grown at 37°C with vigorous shaking until lysis occured. A few drops of chloroform were added, and the lysate clarified by centrifugation prior to titration.

2.4.4 Preparation of phage P1 plate lysates

Preparation of phage P1 plate lysates was as for phage λ except that 10^6 phage were added to 1 ml of late-log phase cells and this was incubated at 37°C for 30 min prior to addition to the top agar. The maltose was omitted, and the $MgSO_4$ was replaced with 2.5 mM $CaCl_2$. The phage buffer was also replaced with the same volume of L-broth + 2.5 mM $CaCl_2$.

2.4.5 Phage P1-mediated transduction

The recipient strain of *E. coli* was grown up to late-log phase in L-broth. The cells were harvested by centrifugation and the bacterial pellet resuspended in 0.1x the original volume of L-broth + 2.5 mM $CaCl_2$. One hundred microlitre aliquots of this 10x culture were mixed with either 0.1 ml of phage P1 stock or 0.1 ml of a 10x dilution of the phage stock. These were incubated at 37°C for 15 min. If prototrophic transductants were to be selected, 0.4 ml of phage buffer was added and 0.2 ml aliquots of the cells plated on the appropriate minimal media agar plates. If the selection was the acquisition of antibiotic resistance then 1 ml of phage buffer was added to the cells, the cells were centrifuged and resuspended in 0.6 ml of LBG. This was incubated at 37°C for 1 h to allow expression of the antibiotic resistance and then 0.2 ml aliquots were plated out on L-broth agar plates containing the appropriate antibiotic. Plates were incubated at a suitable temperature until colonies appeared.

2.5 Protein Techniques

2.5.1 Polyacrylamide gel electrophoresis of proteins

E. coli proteins were routinely separated using SDS–polyacrylamide gel electrophoresis with a discontinuous buffer system (Laemmli,1970). In virtually all cases a 10% resolving gel and 4% stacking gel were employed. For the rapid analysis of proteins a 'Mighty Small' mini–gel apparatus (SE 250) manufactured by Hoeffer Scientific Instruments was used. This used 10 x 5 cm gels, which could be electrophoresed in about 45 min. For better quality gels a larger apparatus (SE 600) made by the same company was used; in this case the gels measured 11 x 14 cm. The composition of typical gel solutions were as follows:

10% Resolving gel.

acrylamide stock solution (40%)	6.25 ml
4x resolving gel buffer	6.25 ml
10% SDS	0.25 ml
7.5% ammonium persulphate (freshly prepared)	0.25 ml
distilled water	12 ml
TEMED	15 µl
Total	25 ml

4% stacking gel.

acrylamide stock solution (40%)	1.0 ml
4x stacking gel buffer	2.5 ml
10% SDS	0.1 ml
7.5% ammonium persulphate (freshly prepared)	0.1 ml
distilled water	6.3 ml
TEMED	10 µl
Total	10 ml

These solutions were made up on ice immediately prior to use, with the ammonium persulphate solution and the TEMED being added last. The resolving-gel solution pipetted between the glass plates separated by 0.75 mm spacers; enough room was left for the stacking gel. For the mini–gel apparatus, the depth of the stacking gel between the bottom of the comb and the resolving gel was about 1 cm, and for

the larger gel was about 2.5 cm. Once the resolving gel had been poured it was layered with isobutanol saturated with 1x resolving-gel buffer and allowed to polymerize for 1 h. The isobutanol was then discarded and the top of the gel was washed with distilled water. The stacking-gel solution was poured on top of the resolving gel, the comb inserted and polymerization allowed to occur. The comb was then removed and the wells washed out with 1x reservoir buffer, which was also used to fill up the buffer chambers of the apparatus. The sample could be loaded onto the gel at this stage.

Samples (typically whole-cell extracts, grown to late log-phase in L-broth, centrifuged and resuspended in a sixth of the original volume of distilled water) were mixed 1:1 with 2x PAGE-loading buffer, boiled for 3 min, and centrifuged in a microfuge for 5 min prior to loading. About 2–5 µl of sample per lane could be loaded onto the mini–gel and 10–30 µl on the larger apparatus. Samples were loaded using a 50 µl Hamilton syringe. Gels were typically electrophoresed at a constant current of 40 mA until the bromophenol blue dye-front had run off the bottom of the gel. Once electrophoresis was complete the glass plates were removed from the apparatus, separated carefully using a plastic wedge and the gel placed in staining solution for 45–60 min with constant gentle agitation. Gels were the transferred into destaining solution and left for anything between 2–24 h. For preservation, the stained/destained gel was soaked in destaining solution plus 5% glycerol for 30 min and dried down on blotting paper using a vacuum gel drier at 80°C for about 1 h.

Solutions used in SDS–PAGE.

Stock acrylamide

37 g acrylamide, 1 g NN' methylene bis–acrylamide, made up to 100 ml with distilled water, filtered and stored at 4°C.

4x stacking-gel buffer (0.5 M Tris)

15.25 g of Tris base, dissolved in 200 ml distilled water, adjusted to pH 6.8 with concentrated HCl, made up to 250 ml, filtered and autoclaved.

4x resolving-gel buffer (1.5 M Tris)

45.5 g of Tris base, dissolved in 200 ml distilled water, adjusted to pH 8.8 with concentrated HCl, made up to 250 ml, filtered and autoclaved.

10x reservoir buffer

30.2 g of Tris base, 144 g of glycine dissolved in 600 ml distilled water, made up to a final volume of 1 litre and filtered. SDS was added to 0.1% in the final 1x buffer.

2x PAGE loading buffer

4x stacking gel buffer		0.125 ml
10% SDS		0.300 ml
50% glycerol		0.200 ml
2–mercaptoethanol		0.050 ml
0.1% bromophenol blue		0.200 ml
Distilled water		0.125 ml
	Total	1.0 ml

Staining solution

9% (v/v) acetic acid, 45% (v/v) methanol, and 0.1% (w/v) Coomassie brilliant blue.

Destaining solution

7% (v/v) acetic acid, and 5% (v/v) methanol.

2.5.2 Western blotting procedures

Western blotting (the transfer of proteins from an acrylamide gel to a membrane) was performed using a BioRad electrophoretic transfer cell. The previously run gel was trimmed down by removing the stacking gel and placed on top of several sheets of blotting paper saturated in protein-transfer buffer (which is 1x protein gel reservoir buffer but without any SDS). A sheet of nitrocellulose saturated in the same buffer was placed on top of the gel excluding any bubbles followed by several more sheets of saturated blotting paper. The whole sandwich was secured in the transfer apparatus and transfer was allowed to continue at 1 amp for 1 h. After this the nitrocellulose was

rinsed in transfer buffer and placed in a heat-sealable bag along with 20 ml of Buffer 1 plus 5% BSA. (Buffer 1 = 0.1 M Tris–HCl (pH 7.6), 0.15 M NaCl). The bag was sealed, avoiding the inclusion of bubbles, and incubated at 65°C for 1 h. The filter was now ready for the application of the primary antibody. The primary antibody (in this case affinity purified anti–GroEL raised in a rabbit) was diluted 1:500 in Buffer 1 and 15 ml of this were used per filter. The solution was poured into a heat-sealable bag containing the filter and sealed as before. The bag and filter were incubated at room temperature with constant agitation for 2 h. After this the filter was removed and washed in three changes of 200 ml of Buffer 1 for 10 min each. The filter was now ready for the application of the secondary antibody. The secondary antibody (in this case biotinylated goat–anti–rabbit IGg) was diluted 1:1000 in Buffer 1. This was added to the filter in a heat-sealable bag using a similar volume of solution and incubation conditions as before. The filter was then removed and washed three times in Buffer 1 as before. The filter was now ready for application of the biotinylated molecule detection system. For this the BRL BluGENE kit was used in an identical fashion to that previously mentioned for the detection of biotinylated DNA molecules (see *2.2.14*).

2.5.3 Preparation of total E. coli *membranes for protein analysis*

For the preparation of membranes from *E. coli* a 250 ml exponential-phase culture of the appropriate strain was grown with vigorous agitation in a 2 litre flask until an OD_{540} of about 0.5 was achieved. The cells were harvested by centrifugation (3600 g, 10 min at 4°C) and the bacterial pellet was resuspended in 5 ml of a solution containing 10 mM Tris–HCl pH 7.6, 5 mM EDTA, and 2 mM 2–mercaptoethanol. The cells were then lysed by sonicating four times for 15 sec each at a setting of 5–6 on the MSE sonicator with chilling on ice between bursts. Unlysed cells and other debris were removed by centrifuging as above and the resulting supernatant ultracentrifuged at 48,000 r.p.m. in a Ti-50 rotor for 1 h at 4°C. The membrane pellet was resuspended in 10 ml of the above buffer and ultracentrifuged as before. The pellet was then resuspended in 1 ml of the same buffer and was then ready for SDS–PAGE analysis of the resultant membrane proteins.

2.5.4 Release of periplasmic β-lactamase by osmotic shock

In order to determine the amount of β-lactamase secreted into the periplasm of *E. coli* the method of Neu and Heppel (1965) was adopted. They used a simple osmotic shock to liberate periplasmic proteins. Twenty five millilitres of L-broth was inoculated with the appropriate strains of bacteria and grown with vigorous agitation until an OD_{540} of 0.5 was achieved. The cells were harvested by centrifugation (3600 g, 10 min at 4°C), the pellet resuspended in 10 ml of bacterial buffer to wash the cells and then recentrifuged. The pellet was then resuspended in 5 ml of a solution containing 20% sucrose, 0.03 M Tris–HCl (pH 8.0), and EDTA added to a concentration of 1 mM. The suspension was incubated in a 100 ml flask on a rotary shaker in a cold room for 10 min after which the cells were harvested as above. The supernatant was removed and the thoroughly drained cell pellet resuspended, this time in 5 ml of distilled water. Again the suspension was mixed on a rotary shaker in a cold room for 10 min and clarified by centrifugation. The resulting supernatant (termed the cold-water wash) could now be assayed for β-lactamase activity.

2.5.5 Determination of β-lactamase activity

Determination of β–lactamase activity was performed using a colorimetric assay system employing nitrocefin (also called 87/312), which was purchased from Glaxo Research. When nitrocefin is hydrolysed by β-lactamases a red colour is produced, which can be quantified using a spectrophotometer (O'Callaghan *et al.*, 1972). In this case osmotic-shock cold-water washes were diluted in 0.01 M phosphate buffer and nitrocefin was added at a final concentration of about 40 $\mu g\ ml^{-1}$. The final reaction volume was usually 1 ml and the OD_{540} of the reaction was measured as a function of time. It should be noted that the reaction was kept dark as this appeared to give better colour development.

2.5.6 Purification of GroEL protein

GroEL protein was purified in its tetradecameric form using a system based on the mehtods of Hendrix (1979) and Bochkareva *et al.* (1992). Five hundred millilitres of L-broth was inoculated with a 5 ml overnight culture of the appropriate strain of *E. coli*. The cells were incubated overnight at 42°C (in order to maximize GroEL protein levels) with vigorous agitation. The resulting culture was centrifuged using a Sorval GS–3 rotor for 15 min at 5000 r.p.m., 4°C, and the cell pellet resuspended in 5 ml of buffer containing 20 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 10 mM $MgCl_2$, 10 mM KCl and 1 mM DTT. This buffer was used throughout the procedure. PMSF was added to a concentration of 200 μg ml^{-1}. The cells were disrupted by sonication allowing 1 min of sonication (in 15 sec bursts with continuous cooling on ice) per gram of cell paste. Typically from 500 ml of culture, 2.5 g of cells were harvested. The total volume was made up to 10 ml with buffer and disrupted cells were clarified by centrifugation using a SS–34 rotor at 20,000 r.p.m. for 1 h at 4°C. The resulting supernatant was further clarified by passing it through a 0.45 μm nitrocellulose filter. This clarified lysate was then fractionated through an S–300 gel filtration column at a pump speed of 0.5 ml min^{-1}. Fractions were collected every 12 min and UV transmission spectroscopy at 260 nm used to determine the amount of absorbing material in each fraction. Because of its size (~800 kDa) GroEL elutes just beyond the void volume of the column. SDS–PAGE analysis was used to determine the fractions with the highest GroEL:other protein ratio. These fractions were pooled and loaded onto a BioRad mono–Q ion exchange column. Proteins bound to this column were eluted by a linear 0–1M NaCl gradient (120 ml) in the above buffer. GroEL eluted at a NaCl concentration of ~0.55 M. After dialysis, SDS–PAGE analysis of the resulting GroEL preparations showed that the protein was very pure (>90%) when the GroEL protein was overexpressed, however when expressed from single-copy vectors preparations were not as clean (~60%). The purified GroEL was stored in the above buffer containing 20% glycerol aliquoted at –20°C.

2.5.7 *Isolation of soluble and insoluble protein fractions from cell lysates*

One hundred millilitres of L-broth was inoculated with 1 ml from an overnight culture of the appropriate strain of *E. coli*. The cells were incubated at the appropriate temperature to late log-phase. The resulting culture was centrifuged using a Sorval GSA rotor for 15 min at 5000 r.p.m., 4°C, and the cell pellet resuspended in 1 ml of buffer containing 20 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 10 mM $MgCl_2$, 10 mM KCl and 1 mM DTT. The cells were disrupted by sonication allowing 1 min of sonication (in 15 sec bursts with continuous cooling on ice) per gram of cell paste. Typically from 100 ml of culture, 0.5 g of cells were harvested. The disrupted cells were clarified by centrifugation in a microfuge at 15,000 r.p.m. for 15 min at 4°C. The resulting supernatant represents the soluble fraction of the cell lysate and could be denatured in PAGE loading buffer and analysed by SDS–PAGE. The pellet was resuspended in 1 ml of the above buffer and centrifuged as before. The pellet was washed three times in this way, and the final pellet represents the insoluble fraction of the lysate. This was resuspended in the above buffer, and could be denatured in PAGE loading buffer and analysed by SDS–PAGE.

CHAPTER 3

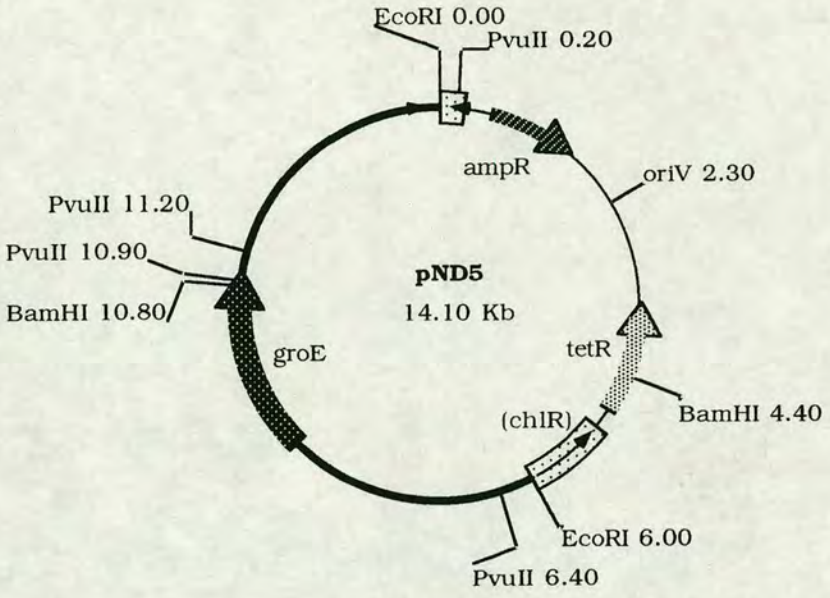
CONSTRUCTING A CLONE WHICH EXPRESSES A TRUNCATED FORM OF GROEL

3.1 Introduction

Suppression of *dnaA* temperature-sensitive mutants by cloned *groE* genes was first reported by Fayet *et al.* (1986), and simultaneously from our laboratory by Jenkins *et al.* (1986). At the time Jenkins was endeavouring to clone the *dnaA* gene. This involved transfecting a *dnaA46*^{ts} strain at its non-permissive temperature with an *Eco*RI library of *E. coli* chromosomal DNA that had been cloned into phage λ. It was expected that colonies that formed at this temperature would be lysogenized with a λ phage carrying the wild-type *dnaA* gene. This strategy, however, was doomed to failure. Although unknown at the time, the *dnaA* gene contains an *Eco*RI restriction site within its coding sequence (Hansen *et al.*, 1984b). Nevertheless, after the transfection, colonies appeared. The λ phages isolated from these transfectants were found to share a DNA insert. It was found that infection with one of these phages (λ*sidA*) allowed only a low frequency of new *dnaA46* lysogens to grow at high temperatures. Further analysis revealed that the cells forming colonies at high temperature were multiple lysogens carrying at least six copies of the λ phage (Fayet *et al.*, 1986) suggesting that growth at 42°C was owing to an extragenic suppressor which was required in high copy.

The chromosomal *Eco*RI DNA insert from λ*sidA* (8.1 kb) was cloned into the high-copy-number plasmid pBR325, yielding plasmid pND5 (Figure 3.1.1). Transformation of pND5 into a *dnaA46* strain allowed growth of the strain at high temperature with survival approaching 100%. It was noticed that these cells now displayed cold sensitivity, growing poorly and with reduced viability at the normally permissive temperature of 30°C. It was discovered that the genes on the 8.1 kb insert, which were responsible for these effects, were the *groE* genes, and that both *groES* and *groEL* were required for suppression of the *dnaA*^{ts} mutation. Jenkins went on to test whether any other DNA replication mutants could be suppressed by overexpression of the GroE proteins; his results were, however, inconclusive.

Figure 3.1.1

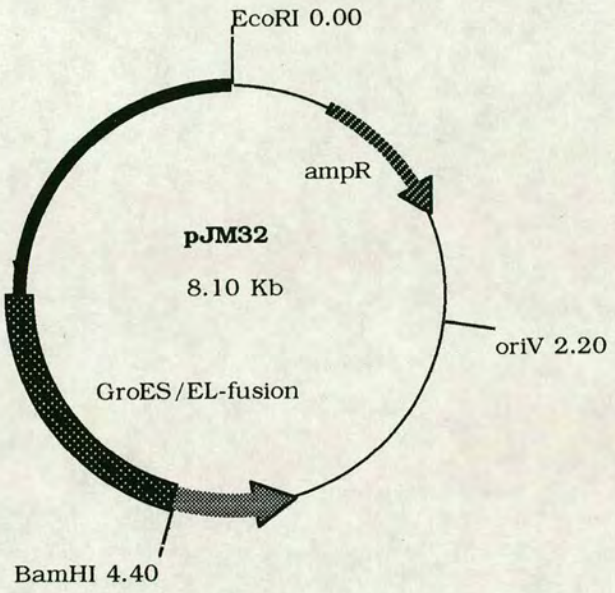


pND5 (Jenkins *et al.*, 1986) was made by ligating the 8.1 kb *Eco*RI DNA fragment encoding the *groE* operon from λ*sidA* into the unique *Eco*RI site of pBR325.

Jenkins' work was continued by March (1988). In the course of his investigations a plasmid derivative of pND5, named pJM32, was constructed. This carried the *groES* gene and DNA encoding the first 495 amino acids (from a total of 548) of GroEL fused out-of-frame to the tetracycline-resistance gene from pBR325. This construct produced a 64 kDa GroEL–fusion protein (Figure 3.1.2). March demonstrated that pJM32, in contrast to pND5, and despite possessing a far lower copy number, suppressed the *dnaA46* mutation; however, it did not complement *groEL*^{ts} mutants. It should be noted that pJM32 was selected in a *dnaA46* strain at a non-permissive temperature, thereby maximizing the selective pressure to overcome the mutant's temperature sensitivity. pJM32 proved a difficult plasmid to work with owing to its extreme instability and low copy number. Large scale plasmid extraction of pJM32 typically yielded only 9 μg of DNA litre^{-1} of culture, in contrast to 1 mg litre^{-1} of

the parent plasmid pBR325. It was also found that pJM32, when incubated overnight without antibiotic selection, cured at a rate of greater than 99% from the *recA* strain DH1. March hypothesized that the instability and low copy number seen with pJM32 was caused by two strong transcriptional promoters reading directly into the plasmid's origin of replication, thus disrupting efficient replication (for a review see Brewer, 1988). Even though pJM32 was present in cells at a much reduced copy number in comparison with pND5 (March estimated a copy-number of 3–4 per cell), it still seemed capable of suppressing *dnaA*^{ts} mutations. March suggested that this was because removal of the carboxyl terminus from the GroEL protein allowed more 'freedom' for the protein to interact with DnaA protein. One region of the removed sequence that seemed particularly interesting was the extreme carboxyl-terminus of the protein. This region contains a striking tandemly repeated glycine.methionine motif (referred to here as the GM motif (Figure 3.1.3)), which is highly conserved amongst almost all the GroEL homologues (see Section 3.2 below). This hydrophobic sequence is unlikely to be folded within the protein and would therefore be available to serve as a membrane anchor. Its removal could possibly have allowed the freedom of movement postulated by March to permit low-gene-dosage suppression. Having myself just joined the laboratory at this point, it was decided that I should begin my studies by seeking to elucidate the function of the carboxyl-terminal GM motif of GroEL, the expectation being that it might prove to be a membrane anchor.

Figure 3.1.2



pJM32, made by John March in this laboratory, was constructed by restricting pND5 with *Eco*RI and *Bam*HI and ligating the resulting fragments. This was transformed into a *dnaA*^{ts} strain at 42°C in order to select for suppressors. The resulting plasmid carried the *groE* operon up to the *Bam*HI site which was fused, out-of-frame, to the tetracycline resistance gene of pBR325. Two of the four pND5 *Bam*HI–*Eco*RI fragments are not included in pJM32. The remaining two fragments are inverted relative to their orientation in pND5.

Figure 3.1.3 The GM motif of the *E. coli* GroEL carboxl-terminus.

...AAA AAC GAT GCA GCT GAC TTA GGC GCT GCT GGC GGT ATG GGC
Lys Asn Asp Ala Ala_{530} Asp Leu Gly Ala Ala Gly Gly Met Gly

GGC ATG GGT GGC ATG GGC GGC ATG ATG TAA TTG...
Gly_{540} Met Gly Gly Met Gly Gly Met Met_{548} *

3.2 Evaluating conservation of the carboxyl-terminal GM motif of GroEL

The sequence data of Hemmingsen *et al.* (1988) first showed that the carboxyl-terminus of the *E. coli* GroEL protein consisted of a string of glycine–methionine amino acids. March (1988) carried out a computer search that demonstrated that many GroEL proteins from a variety of sources also show this motif. In order to extend this (short) list (performed in 1988) a further search was carried out in 1992. A search of the SWISSPROT data base (Version 23) was performed, utilizing the Wisconsin GCG package program (Genetics Computer Group, 1991), using the keywords 'GroEL', 'HSP60', 'chaperonin' and 'cpn60' in order to determine a current list of all available GroEL-like protein sequences. Many further HSP60s have been sequenced since 1992; the list is thus not complete. The carboxyl-terminal amino acids of all GroEL-like proteins found are shown in Table 3.2.1. It can clearly be seen that, with the exception of the chloroplast- located RSBPs (which do have considerable sequence identity with *E. coli* GroEL as a whole), most of the GroEL-like proteins contain a carboxyl-terminal motif rich in glycine and methionine residues. The list demonstrates the highly conserved nature of this motif, from which its importance to GroEL-type proteins can be inferred. Although sharing a GM motif, GroELs originating from different organisms show variations in the exact sequence and length of the motif. For example, extra glycine residues are sometimes seen in the repeated unit, resulting in a GGGM motif; proline residues are sometimes present, and phenylalanine is often seen as the terminal amino acid. This sequence variability suggests that the GM tail is probably not involved in a sequence-specific biological reaction, but rather one in which the chemical attributes (to be discussed below) of this type of sequence are important, although sequence divergence elsewhere in the protein may compensate for mild sequence divergence within the GM motif. The motif is present right across the evolutionary spectrum (higher plant HSP60s from mitochondria do contain carboxyl-terminal GM motifs) implying that the motif either confers an advantage to the protein, and is therefore maintained, or is vital in certain situations to protein function.

Table 3.2.1 HSP60 Computer search (carboxyl-termini)

HSP60s

A. proteus symbiont	AGAGDMGGMGGMGGMGGMGMM* 551[a]	Ahn *et al.* (1991)
Synechocystis sp.	PAPPAMPDMGGMGGMGGMGGMGMM* 551	Chitnis and Nelson (1991)
L. micdadei	KKEEPMGAGEMGGMGGMGGMGGMM* 546	Hindersson et al. (1991)
L. pneumophila	KKEEGVGAGDMGGMGGMGGMGGMM*547	Sampson *et al.* (1990)
E. coli	PKNDAADLGAAGGMGGMGGMGGMM* 548	Hemmingsen *et al.* (1988)
C. burneti	KKEESMPGGGDMGGMGGMGGMGGM* 551	Vodkin and Williams (1988)
R. norvegicus	IPKEEKDPGMGAMGGMGGGMGGGMF* 573[m]	Venner and Gupta (1990a)
M. musculus	IPKEEKDPGMGAMGGMGGGMGGGMF* 573[m]	Venner and Gupta (1990b)
H. sapiens	IPKEEKDPGMGAMGGMGGGMGGGMF* 573[m]	Jindal et al. (1989)
C. griseus	IPKEEKDPGMGAMGGMGGGMGGGMF* 573[m]	Picketts *et al.* (1989)
B. abortus	AELPKKDAAPAGMPGGMGGMGGMDF* 546	Gor and Mayfield (1992)
S. cerevisiae	AIVDAPEPPAAAGAGGMPGGMPGMPG* 570[m]	Johnson *et al.* (1989)
A. pisum symbiont	KEDKSSDSSSSPAGGMGGMGGMM*548	Ohtaka *et al.* (1992)
M. leprae	EAVVADKPEKTAAPASDPTGGMGGMDF* 541	Mehra *et al.* (1986)
B. burgdorferi	AITDIKEEKNTSGGGGYPMDPGMGMM* 545	Shanafelt *et al.* (1991)
M. tuberculosis	EAVVADKPEKEKASVPGGGDMGGMDF* 540	Shinnick (1987)
R. tsutsugamushi	DHEEDNNTGNRSGGGVGGGHHGGMG* 551	Stover *et al.* (1990)
A. nidulans	ECIVVDKPEPKEKAPAGAGGGMGDFDY* 544[m]	Webb *et al.* (1990)
C. perfringens	LTTEAAVADIPEKEMPQGAGMGMDGMY*539	Rusanganwa *et al.* (1992)
C. psittaci	TTELLALIADIPEEKSSSAPAMPGAGMDY*544	Morrison *et al.* (1989)
C. trachomatis	LLTTEALIAEIPEEKPAAAPAMPGAGMDY*544	Morrison *et al.* (1990)

Rubisco subunit-binding proteins (RSBPs)

B. napus (α-subunit)	LTTQAIVVDKPKPKAPTAAPPQGLMV* 545	Martel *et al.* (1990)
B. napus (β-subunit)	HAASVAKTFLMSDCVVVEIKEPEPVP* 588	Martel *et al.* (1990)
R. communis (α)	MTDKYENLVEAGVIDPAKVTRCALQN* 495	Hemmingsen *et al.* (1988)
T. aestivum (α)	LTTQAIVVEKPKPKPKVAEPAEGQLSV* 543	Hemmingsen *et al.* (1988)

This table shows the carboxyl-termini of HSP60 molecules retrieved from the SWISSPROT protein data base. The sequences have been arranged according to the lengths of the GM motifs, which are underlined. Note that none of the RSBPs contains a GM motif.
a. Total number of amino acids in the protein.
m. Mitochondrial protein.
*. Denotes the carboxyl-terminus of the protein.

Using the *E. coli* GM motif as a searching sequence, the SWISSPROT data base was scanned to determine whether any other non-HSP60 proteins contained a similar motif. This search identified several proteins containing repeated sequences rich in glycine (see below), but the only protein species that showed a true GM motif were members of the eukaryotic HSP70 protein family (Table 3.2.2). (No HSP70 sequences from prokaryotic organisms were found to contain this motif.) In these cases the GM motif is located near but not at the very end of the protein sequence (as is seen with HSP60s). It is uncanny that such a sequence should only be found in two distinct classes of HSPs.

Table 3.2.2 GM motifs found in HSP70 molecules.

LD mildew	GAAGGMPGGMPGMPGGMPGMPGGMPGMPGGMPGGMPGGMPGGWPPCW-	675[a]
T. brucei	QGMGGGDGPGGMPEGMPGGMPGGMPGGMGGGMGGAAASSGPKVEEVD*	661
P. falc.	GGMPGGMPGGMPGGMPSGMPGGMNFPGGMPGAGMPGNAPAGSGPTVEEVD*	681
D. melano.	CNPIITKLYQGAGFPPGGMPGGGGGMPGAAGAAGAAGAGGAGPTIEEVD*	651
H. sapiens	EKVCNPIITKLYQSAGGMPGGMPGGFPGGGAPPSGGASSGPTIEEVD*	646

HSP70 sequences found in the SWISSPROT protein data base by searching with the GM motif.
LD, lettuce downy.
a. Total number of amino acids in the protein.
*. Denotes the carboxyl-terminus of the protein.

March (1988) carried out extensive data base searches for proteins containing glycine-rich repeats using the consensus sequence GG(GG)X GG(GG)X GG(GG)X GG(GG)X, where X represents any amino acid. Of the 200 'best-fitting' protein sequences uncovered most fell into one of three catagories; keratinous proteins, viral capsid proteins, and colicins. Keratins and cytokeratins are a subclass of a large group of proteins known as intermediate filaments which fulfil a cytoskeletal role in the cell and are therefore structural proteins (for a review see Steinert *et al.*, 1985). It has been postulated that the glycine-rich domains in such proteins could be acting as recognition

sequences or interaction sites involved in macromolecular assembly (polymerization). The glycine-rich domains are inferred to be hydrophobic, which may suggest a role in membrane attachment. This could account for their presence in viral capsid proteins and in colicins. Indeed, such a sequence is present in the capsid protein B of bacteriophage λ, which is known to interact with GroE proteins during λ infection in *E. coli* (Murialdo, 1979).

In summary it can be seen that the GM motif of HSP60s is highly conserved within that protein family. Analysis of proteins containing glycine-based repeated motifs has shown that many of these proteins are structural, with the glycine domains possibly acting as sequences that interact with similar sequences allowing polymerization of molecular filaments and/or membrane attachment. Other proteins containing such glycine-rich motifs (viral capsid proteins and colicins) interact with cell membranes, although it is not known whether the glycine domains are actually involved in membrane recognition and/or interaction.

3.3 Construction of GroEL lacking the GM motif

Plasmid pJM32 encodes a fusion protein comprising the first 495 amino acids of GroEL and out-of-frame amino acids derived from the *tet* sequence. This protein appeared to be able to suppress *dnaA* temperature-sensitive mutations, although unable to replace GroEL. Since our working hypothesis was that these properties were owing to the absence of the GM motif, we decided to mutate *groEL* so that it would encode a protein with a simple carboxyl-terminus truncation. This was achieved by utilizing a fortuitously positioned *Pvu*II restriction site just upstream of the GM tail (Figure 3.3.1). Plasmid pIR88 was used for constructing the truncation, rather than our other *groE*-containing plasmid pND5, which carries exactly the same 8.1 kb *Eco*RI insert. pIR88 has three *Pvu*II restriction sites rather than pND5's four, and so partial digestion would yield fewer products thus making the cloning easier (Figure 3.3.2). pIR88 was partially restricted with decreasing amounts of *Pvu*II and the products of the digestions were electrophoresed through an agarose gel (Figure 3.3.3).

Figure 3.3.1 The DNA and amino acid sequence of *groEL* carboxyl-terminus showing the *Pvu*II restriction site and the Universal Translation Terminator.

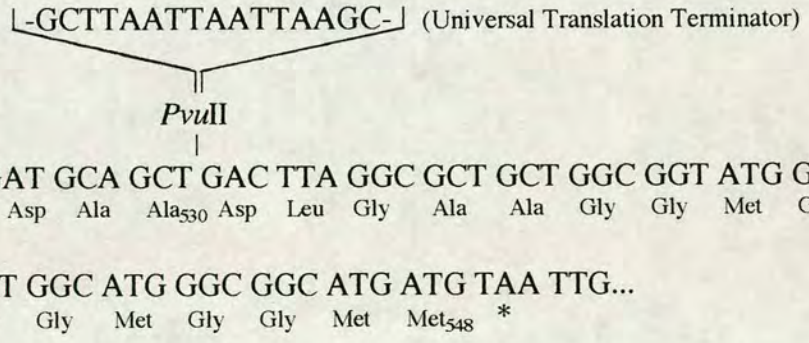
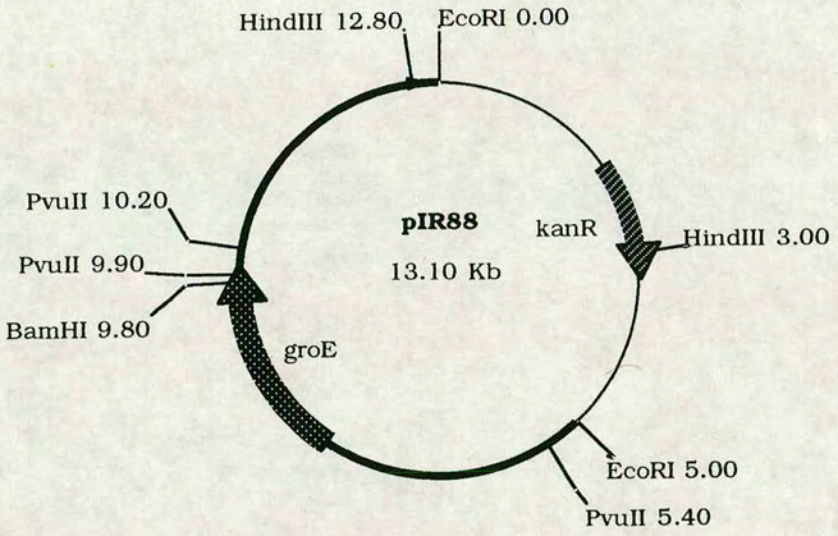
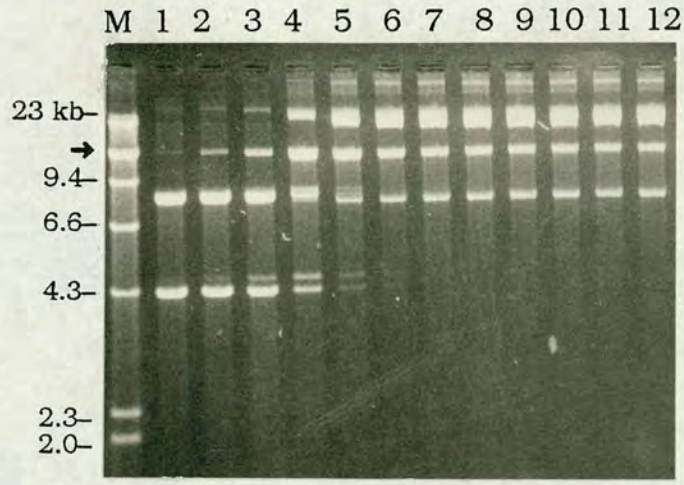


Figure 3.3.2



pIR88 (Oliver, unpublished) was constructed by ligating the 8.1 kb *Eco*RI DNA fragment encoding the *groE* genes from pND5 into the unique *Eco*RI site in pVH1.

Figure 3.3.3 Partial digestion of pIR88 with *Pvu*II.



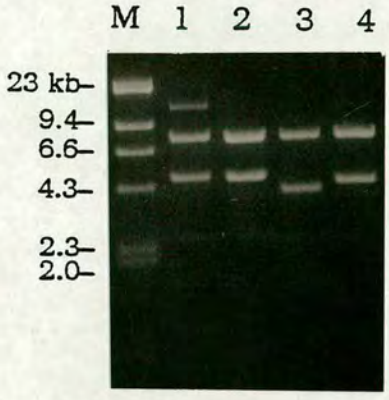
M	λ *Hin*dIII markers plus pIR88 linearised with *Bam*HI (➔)
1	pIR88 *Pvu*II (fully restricted)
2-11	pIR88 Partial digestion products (see below)
12	pIR88 unrestricted

2 units of *Pvu*II were added to 2 μg of pIR88 DNA in a reaction volume of 50 μl. 25 μl of this were diluted into 25μl of a buffered solution containing 1 μg pIR88. This dilution was repeated a further nine times so that each reaction contained 1 μg DNA and twofold sequential reductions in the amount of *Pvu*II. These reactions were incubated for 40 min at 37°C and the products electrophoresed through a 0.8% agarose gel. The linearized plasmid bands were extracted from lanes 6–9 and used for the construction of pHC18.

The linearised plasmid band was removed from the gel and the DNA was ligated to a 20-fold molar excess of the Universal Translation Terminator (Pharmacia; Prod. no. 27-4890). This is a blunt ended, palindromic, double-stranded 16 bp oligonucleotide with TAA STOP codons in all three possible reading frames (see Figure 3.3.1). Plasmids containing the insertion could be identified by screening the DNA for loss of the *Pvu*II restriction site. Without this screen it would have been difficult to confirm insertion as the oligonucleotide carries

no distinguishing restriction endonuclease sites of its own, and its high AT content makes it highly unsuitable as a DNA hybridization probe. The products of the linearized plasmid/Universal Translation Terminator ligation were transformed into *E. coli* strain DH1 (Hanahan, 1983) and plated onto L-agar plates containing kanamycin. In the correct construction the large 4.2 kb *Pvu*II fragment of pIR88 increased in size to 4.5 kb owing to loss of the internal *Pvu*II site (Figure 3.3.4). This plasmid was named pHC18 (Figure 3.3.5). SDS–PAGE analysis of the protein product showed that the cells were producing a truncated form of GroEL with a slightly increased electrophoretic mobility in comparison with the wild-type protein (Figure 3.3.6).

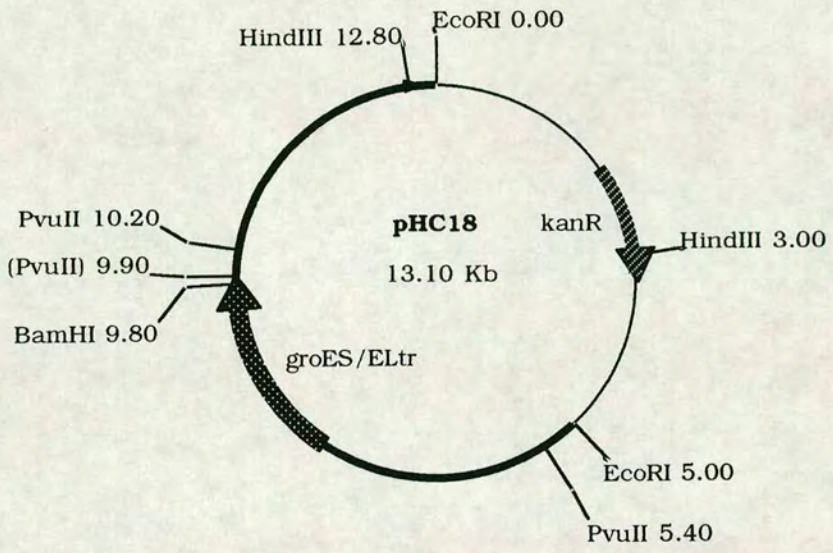
Figure 3.3.4 Restriction of pIR88 and pHC18 DNA.



M	λ *Hin*dIII markers
1	pIR88 *Eco*RI
2	pHC18 *Eco*RI
3	pIR88 *Pvu*II
4	pHC18 *Pvu*II

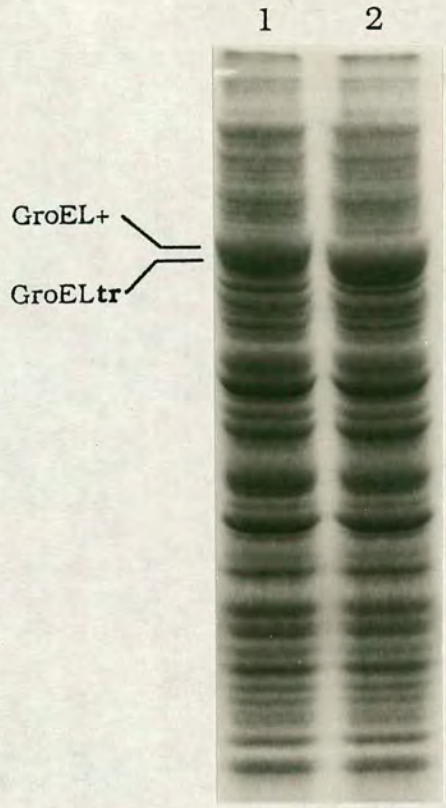
Lanes 1 and 2 show the 8.1 kb chromosomal *Eco*RI fragment encoding the *groE* genes. Lanes 3 shows the 4.2 kb *Pvu*II fragment of pIR88 and lane 4 shows this band shifting to 4.5 kb owing to insertion of the Universal Translation Terminator in the *Pvu*II site just upstream of the GM motif-encoding sequence in *groEL*.

Figure 3.3.5



pHC18 was made by ligating the Universal Translation Terminator (a 16 bp double-stranded oligonucleotide with UAA stop codons in all three reading frames) into the *Pvu*II site within the 3' region of the *groEL* coding sequence. During the construction of pHC18 another plasmid named **pHC23** was isolated. In pHC23 a 300 bp *Pvu*II deletion occurred, it therefore carries no *groEL*-specific DNA downstream of the *Pvu*II site. Plasmid pHC23 was used to construct the *groE* deletion strain described in *Chapter 4*. Both plasmids produce identically truncated forms of GroEL.
(PvuII) denotes the *Pvu*II sites lost owing to insertion of the Universal Translation Terminator.

Figure 3.3.6 SDS–PAGE protein analysis on cells carrying pIR88 and pHC18.



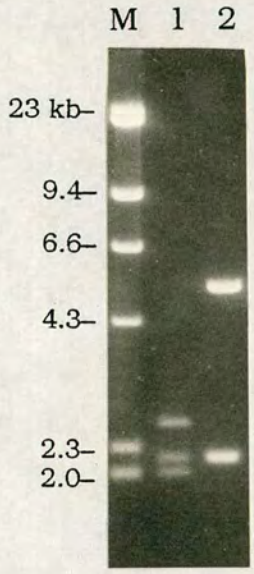
1 DH1 pIR88 whole-cell extract
2 DH1 pHC18 whole-cell extract

Cultures of DH1 carrying each plasmid were grown in L-broth to mid-log phase at 37°C. These were then shifted to 42°C and grown for a further hour at this temperature. Cells were then prepared as detailed in *Materials and Methods* and the samples subjected to SDS–PAGE using a 10% resolving gel. Note the increase in migration of $GroEL_{tr}$ produced from cells carrying pHC18 relative to $GroEL^{+}$ from cells carrying pIR88.

pIR88 and pHC18 are ColD, kanamycin-resistant plasmids. It was decided that ampicillin-resistant ColE plasmids encoding both wild-type and truncated GroELs would be useful. To make these the *groE* operon was cloned from pGroESL (Goloubinoff *et al.*, 1989b) on a 2.1 kb *Eco*RI–*Hin*dIII fragment into the *Eco*RI–*Hin*dIII sites of pJF118EH (Figure 3.3.7). The resulting 7.4 kb plasmid, designated pGT3270

(Figure 3.3.8), was shown to complement *groES* and *groEL* temperature-sensitive mutations for both growth at 42°C and the plating of phage λ, and to suppress *dnaA* temperature-sensitive mutations.

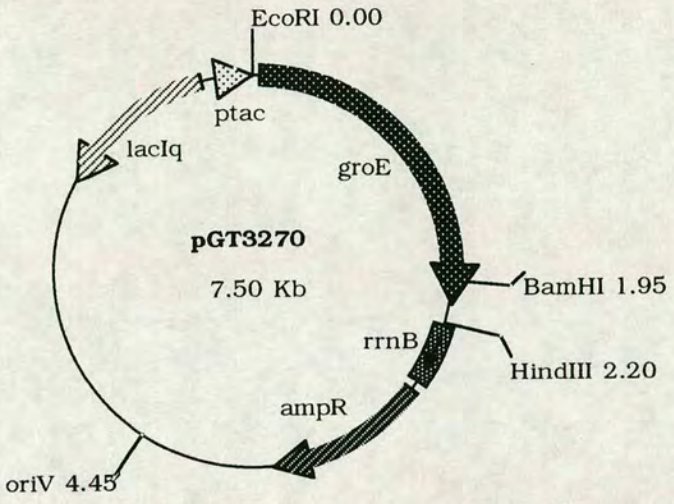
Figure 3.3.7 Restriction of pGroESL and pGT3270.



M	λ *Hin*dIII markers
1	pGroESL *Eco*RI / *Hin*dIII
2	pGT3270 *Eco*RI / *Hin*dIII

Lane 1 shows the three bands generated by restricting pGroESL with *Eco*RI and *Hin*dIII. The middle 2.1 kb band contains the *groE* operon. This band was cloned into pJF118EH to give pGT3270. Lane 2 shows pGT3270 restricted with *Eco*RI and *Hin*dIII resulting in release of the 2.1 kb *groE* fragment from the 5.3 kb backbone of pJF118EH.

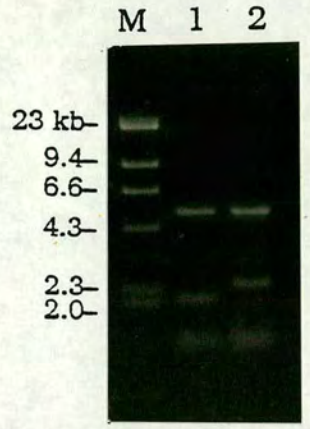
Figure 3.3.8



pGT3270 was constructed by ligating the 2.2 kb *Eco*RI–*Hin*dIII DNA fragment containing the *groES* and *EL* genes from pGroESL (Goloubinoff *et al.*, 1989b) into the *Eco*RI and *Hin*dIII sites in pJF118EH.

pGT3270 was restricted with *Bam*HI and *Hin*dIII and into these sites were ligated the 5 kb *Bam*HI–*Hin*dIII fragments from pIR88 and pHC18 which carry the carboxyl end of the *groEL* gene and the downstream DNA. Restriction analysis verified these constructs (Figure 3.3.9). The new pGT3270 derived plasmids containing fragments from from pIR88 and pHC18 were called pGTIR88 and pGTHC18, respectively (Figure 3.3.10). As before their protein products were analysed by SDS–PAGE (Figure 3.3.11).

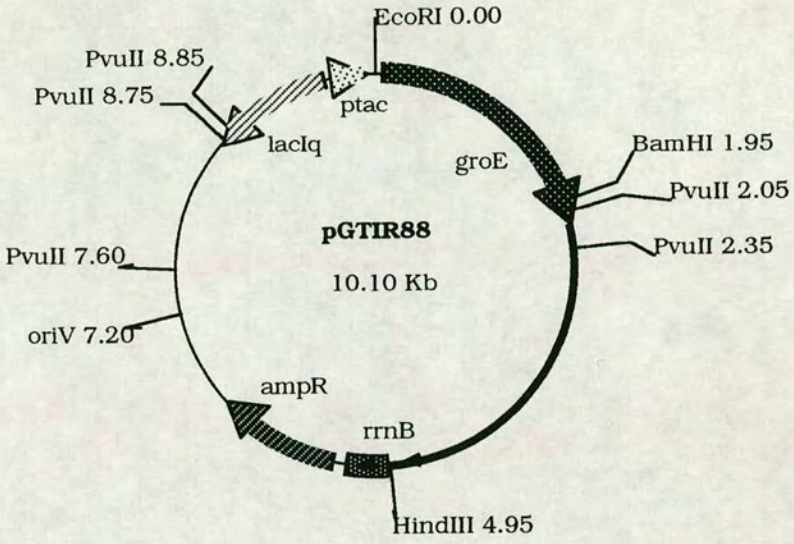
Figure 3.3.9 Restriction of pGTIR88 and pGTHC18.

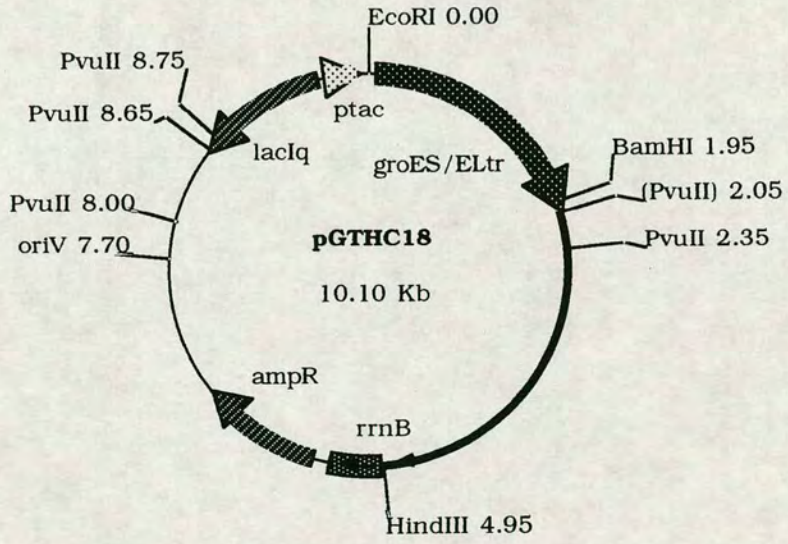


M	λ *Hin*dIII markers
1	pGTIR88 *Eco*RI / *Pvu*II
2	pGTHC18 *Eco*RI / *Pvu*II

This gel shows the increase in size of the 2.1 kb *Eco*RI–*Pvu*II fragment from pGTIR88 to the 2.4 kb fragment seen in pGTHC18 due to insertion of the Universal Translation Terminator into the *Pvu*II site just upstream of the GM motif-encoding sequence of *groEL*.

Figure 3.3.10





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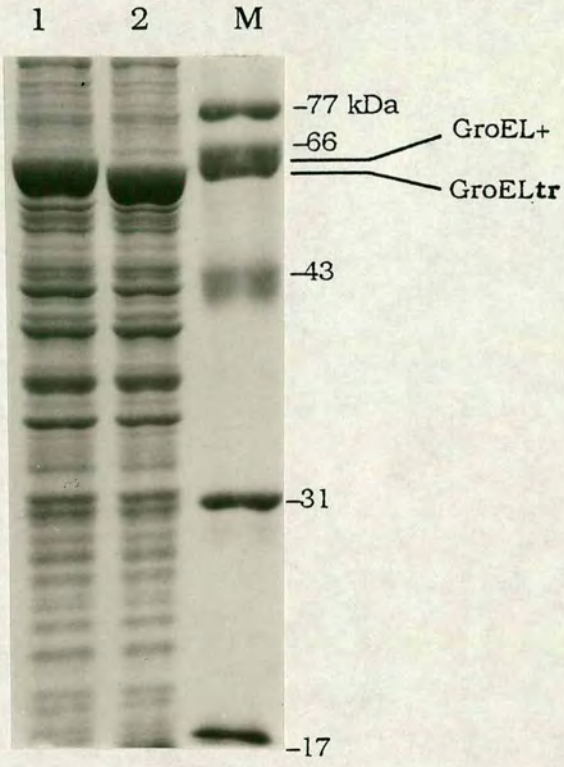
pGTIR88 and **pGTHC18** were constructed by ligating the 3 kb *Bam*HI–*Hin*dIII DNA fragments encoding the distal end of the *groEL* (and *groEL*$_{\mathbf{tr}}$) genes from pIR88 and pHC18, respectively, into the *Bam*HI–*Hin*dIII sites of pGT3270 carrying *groES* and *groEL* up to the *Bam*HI site.
(PvuII) denotes the *Pvu*II site lost due to insertion of the Universal Translation Terminator and thus encoding GroEL$_{\mathbf{tr}}$.

Figure 3.3.11 SDS–PAGE protein analysis on cells carrying pGTIR88 and pGTHC18.



1	DH1 pGTIR88 whole-cell extract
2	DH1 pGTHC18 whole-cell extract
M	Molecular weight markers

Cultures of DH1 carrying each plasmid were grown in L-broth to mid-log phase at 37°C. These were then shifted to 42°C and grown for a further hour at this temperature. Cells were then prepared as detailed in *Materials and Methods* and the samples subjected to SDS–PAGE using a 10% resolving gel. Note the increase in migration of $GroEL_{tr}$ produced from cells carrying pGTHC18 relative to $GroEL^{+}$ from cells carrying pGTIR88.

3.4 Construction of isogenic *groE* strains

In order to compare differences between the various *groE* mutations it was decided to move the mutations from their original strain backgrounds into the same isogenic background. This was achieved by making P1 lysates on the *groE* mutant strains, obtained from C. Georgopolous and R. Hendrix, and using these to transduce a recipient strain to the *groE*^{ts} phenotype. The *groE*^{ts} mutants in our collection carry one of the alleles *groES30*, *groES131*, *groES619*, *groEL44*, *groEL100* or *groEL140*. The recipient strain chosen was PC0698, which is a *purA* strain requiring adenine for growth on minimal medium. The *groE* operon, located at 93.5 minutes on the *E. coli* genetic map, is about 15% cotransducible with the *purA* gene. P1 lysates prepared on the *groE* mutant strains were used to transduce PC0698 from *purA* to *purA*$^{+}$, and the *purA*$^{+}$ progeny screened to identify the 15% that had inherited the mutant *groE*^{ts} allele. Fifty *purA*$^{+}$ clones from each transduction were screened on minimal agar plates at 30 and 42°C. In all cases temperature-sensitive clones were found at the expected frequency. The *groE* phenotype was confirmed by demonstrating resistance to bacteriophage λ at permissive temperatures. Bacterial lawns were made using a temperature-sensitive clone from each transduction (plus one *purA*$^{+}$, temperature-resistant clone as a control) onto which serial dilutions of wild-type phage λ and λ*sidA* (*groE*$^{+}$) were spotted. The plates were incubated overnight at 37°C and then checked for the presence of plaques. The wild-type phage λ did not make plaques on any of the strains except the temperature-resistant control, but the *groE* complementing λ*sidA* formed plaques on all the strains. This demonstrated that the strains isolated did indeed carry the mutant *groE*^{ts} alleles (Table 3.4.1).

Table 3.4.1 *GroE*^{ts} strain constructions.

Donor strain	Allele	New strain	Colony size		Phage Sensitivity	
			30°C	42°C	λ_{wt}	$\lambda sidA$
	groE^{+}	NL1[a]	+++	+++	+++	+++
HX24	*groES30*	NL30	++	-	-	+++
AG31	*groES131*	NL131	+++	+[b]	+[b]	+++
HX39	*groES619*	NL619	++	-	-	+++
HX369	*groEL44*	NL44	++	-	-	+++
AG40	*groEL100*[c]	NL100	++	-	-	+++
CG714	*groEL140*[c]	NL140	++	-	-	+++

GroE^{ts} strains constructed as described in text.
+++ , well-formed colonies/plaques; - , no visible colonies/plaques.
a. A *purA*$^{+}$, *groE*$^{+}$ clone produced during the transduction of PC0698 using the P1 lysate from AG31
b. A leaky allele showing some growth at the non-permissive temperature and some lysis with λ_{wt}.
c. These alleles have been shown to have the same mutation (Zeilstra-Ryalls *et al.* 1991a).

One strain, NL131 (*groES131*), was found to be leaky both for 42°C survival (some growth being observed) and plating λ phages, however presence of the *groE* genes on λ*sidA* improved plaque formation dramatically and so it seemed likely that NL131 was indeed a *groE* mutant. Some of these alleles have recently been sequenced revealing that *groEL100* and *groEL140* carry the same mutation (serine to phenylalanine) at codon 201 even though they were isolated separately. The *groEL44* mutation has a glycine for glutamine substitution at codon 191 as well as several base substitutions in non-coding regions of the operon. *GroES30* has an alanine to serine substitution at codon 31, as well as a mutation in the Shine–Dalgarno sequence (Zeilstra-Ryalls *et al.*, 1991a). All the *groE* mutants were also shown to be complemented for high temperature growth and phage sensitivty by pND5. The *groEL*^{ts} strains NL44, NL100 and NL140 were also transformed with plasmids encoding HSP60s from different sources. These were pHSP60 encoding *S. cerevisiae* HSP60 (given by

R. Hallberg); pMyco65, encoding mycobacterial 65kDa antigen (gifted by D. Young) and pRubSBP encoding rubisco subunit-binding protein-α from wheat (gifted by J. Ellis). None of the proteins expressed from these plasmids was able to complement any of the *groEL* mutations described for either high temperature survival or phage growth.

Two of the strains, NL30 (*groES30*) and NL44 (*groEL44*), which were to be the most frequently used *groE*^{ts} alleles in this work, were mated with the Hfr strain JC10-240, which carries the *recA56* mutation closely linked to a tetracycline-resistant Tn*10* within 5 minutes of the origin of transfer, to produce two *groE*^{ts}, *recA56* strains. *RecA* derivatives of the *groE*^{ts} strains were required for the analysis of *groE*-derived genes carried on plasmids to avoid homologous recombination. These strains were designated NL302 and NL441.

3.5 Complementation and suppression analysis

Plasmids carrying either wild-type (*groEL*$^+$) or truncated (*groEL*$_{tr}$) forms of the gene were tested for their ability to both complement *groEL* and suppress *dnaA* temperature-sensitive mutations. In light of the previous experiments performed by J. March it was expected that the truncated form of *groEL* would be unable to complement *groEL* mutations but would be able to suppress *dnaA* mutations in an allele-specific manner in combination with *groES*, i.e. similar to the results obtained with pJM32.

3.5.1 Complementation of groEL

Both sets of plasmids (ColD, Kan^R; pVH1, pIR88, pHC18 and ColE, Amp^R; pJF118, pGTIR88, pGTHC18) were transformed into *groE*-mutant strains at the permissive temperature of 30°C. Transformants were then tested for their ability to grow on plates at the non-permissive temperature of 42°C, and to support phage λ, T4 and T5 propagation at permissive temperatures. It can be seen that the truncated form of *groEL* was able to complement all the *groEL* mutations in our collection as efficiently as did the wild-type gene for both high temperature growth and phage development (see Table 3.5.1). This was a completely unexpected result as it was not consistent with the behaviour of pJM32. Furthermore, it implied that the GM tail did not play an important, let alone vital, role in the protein's normal function.

Table 3.5.1 Complementation of *groEL44* by *groEL*$^+$ and *groEL*$_{tr}$.

Strain	Plasmid	Colony Size		Phage Sensitivity					
				λ		T4		T5	
		30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C
NL441 (*groEL44 recA56*)	pJF118	++	-	-	na	-	na	-	na
	pGTIR88 (*groEL*$^+$)	++	+++	++	+++	++	+++	++	+++
	pGTHC18 (*groEL*$_{tr}$)	++	+++	++	+++	++	+++	++	+++
	pVH1	++	-	-	na	-	na	-	na
	pIR88 (*groEL*$^+$)	++	+++	++	+++	++	+++	++	+++
	pHC18 (*groEL*$_{tr}$)	++	+++	++	+++	++	+++	++	+++

NL441 was transformed with the given plasmids at 30°C. Resulting clones were (i) streaked out on L-agar+Amp or Kan, and (ii) used to make bacterial lawns for the plating of phages, both at 30 and 42°C. All the *groE*^{ts} alleles in our collection were tested with similar results.
+++, well formed colonies/plaques; -, no visible colonies/plaques
na, not applicable.

3.5.2 *Suppression of* dnaA

The ColD, Kan^R set of plasmids were transformed into MM185 (*dnaA46*) at 30 and 42°C (Table 3.5.2). As expected, no pVH1 transformants of this strain were obtained at the higher temperature and the 30°C transformants would not grow at 42°C. pIR88 transformed efficiently into MM185 at 42°C but inefficiently at 30°C, which is also expected since the high temperature suppression of *dnaA* mutations by overexpression of the GroE proteins is also associated with a cold sensitivity at the lower temperature. When pHC18 was transformed into MM185 it was found that at 30°C the transformation was efficient, but at 42°C it was rather inefficient in comparison with pIR88.

Table 3.5.2 Suppression of *dnaA*46 by *groEL*$^+$ and *groEL*$_{tr}$.

Strain	Plasmid	Colony Size		Transformation efficiency/μg DNA	
		30°C	42°C	30°C	42°C
MM185 (*dnaA46*)	pVH1	+++	-	$3.0x10^5$	0
	pIR88 (*groEL*$^+$)	+[a]	+++	$1.1x10^3$	$4.0x10^5$
	pHC18 (*groEL*$_{tr}$)	+++	++[b]	$2.7x10^5$	$7.7x10^2$

MM185 was transformed with 100 ng of plasmid DNA and plated out at 30 and 42°C. The resulting colonies were counted and the transformation efficiencies calculated.
+++, well formed colonies; -, no colonies visible.
a. Poor growth due to the cold sensitivity exhibited by this combination
b. Good sized colonies but greatly reduced in numbers compared to growth at 30°C (as can be seen from the transformation efficiency).

The 30°C transformants did not restreak well when plated at 42°C; it was found that on average less than 1% of the total number of cells plated were able to form colonies at 42°C. This compared to a value approaching 100% for cells containing pIR88. It was found, however, that the MM185(pHC18) that managed to grow at 42°C were now 100% viable when rechallenged at the high temperature. In addition they now showed poorer growth at 30°C (although this was not nearly as dramatic as the cold sensitivity seen with MM185(pIR88)) suggesting that they formed a genetically distinct sub-population in contrast to cells that had not been exposed to the higher temperature. It was also found that these cells no longer overproduced the GroEL$_{tr}$ protein and that plasmid preparations from these cells contained very little DNA in comparison to both MM185(pHC18), which had never been exposed to the higher temperature, and MM185(pIR88) from 42°C. These results suggested that the suppression seen with pHC18 was different from that seen with pIR88 and seemed to occur only when pHC18 had a reduced copy number. Since pJM32 mediated suppression also occurred with

reduced plasmid copy number, it was thought that suppression of *dnaA*^{ts} mutations by GM-lacking forms of the GroEL protein might rely upon a low gene dosage, and that a mutation may have occured in the MM185(pHC18) suppressed cells that limited the amount of truncated GroEL available. If this were the case then a *dnaA*^{ts} strain, which because of an additional mutation maintains plasmids at a reduced copy number, should be suppressible by pHC18. JM21 (*dnaA46*, *pcnB21*) transformed with pHC18 would be such a strain, but transformation of JM21 showed similar results to those gained using MM185 suggesting that a different explanation was more likely. It was further reasoned that if a specific chromosomal mutation of MM185(pHC18) was allowing the suppression of the *dnaA46* mutation then curing the strain of the plasmid and subsequent retransformation with pHC18 should produce levels of suppression approaching 100%. Attempts to cure the plasmid from pHC18-suppressed MM185, and pHC18-unsuppressed MM185 (never exposed to high temperature, and still showing pHC18 present in high-copy number) provided unexpected results that changed the direction of this project.

The strains were incubated for three days in L-broth without antibiotic plasmid selection at 30°C. A dilution of the cells was plated out to give single colonies, these were then patched onto plates with and without kanamycin. The results showed that pHC18 had been cured from 75% of the unsuppressed cells, but not at all from the suppressed ones, suggesting that the plasmid had become chromosomally located in these cases, and that this integration was responsible for the *dnaA* suppression (Table 3.5.3).

Table 3.5.3 Curing of plasmid pHC18 from MM185.

Strain	No. of Colonies -Kan	No. of Colonies +Kan	Curing rate (%)
MM185 pHC18 $_{(30°C)}$[a]	100	25	75
MM185 pHC18 $_{(42°C)}$[b]	100	100	0

Strains were inoculated at 30°C without kanamycin plasmid selection. These were grown up overnight, diluted 1:5000 in fresh medium and the cycle repeated a further two times. A dilution of each culture was plated onto L- agar, yielding single colonies, 100 of which were patched onto plates with and without kanamycin. The plates were incubated overnight at 30°C and growth compared.
a. Unsuppressed strain never exposed to 42°C.
b. Suppressed strain isolated at 42°C.

There are several known mechanisms of *dnaA* suppression. One of these involves bypassing the need for the DnaA-dependent origin of replication by initiating chromosomal replication from a DnaA-independent origin (see Introduction). It is believed that this may be what is happening here, with pHC18 integrating into the chromosome by RecA-mediated recombination owing to the homology that exists between the plasmid insert and the chromosome, enabling the plasmid origin to direct DNA replication in the absence of DnaA protein. During integrative suppression of *dnaA*^{ts} the closer the inserted plasmid is to *oriC* (located at 84 minutes), the better the chromosomal replication from the plasmid origin will be, and so insertion with recombination near the *groE* operon at 93.5 minutes should result in fairly efficient chromosomal replication (Louarn *et al.*, 1982). For integrative suppression to occur, homologous recombination between plasmid and chromosome, and thus RecA, is required. When a *dnaA*^{ts}, *recA* strain was transformed with *groE* plasmids it was found that unlike pIR88 and pGTIR88, plasmids carrying GroEL$_{tr}$ (in combination with GroES) could not suppress the temperature-sensitive mutation suggesting that pJM32 enabled *dnaA*^{ts} strains to grow by integrating at 42°C. Furthermore the strains showed no cold sensitivity, confirming that high temperature

suppression and cold sensitivity are different manifestations of the same effect (Table 3.5.4).

To further extend these results pHC18 was transformed into all the temperature-sensitive *dnaA* mutants in our collection. It was found that the truncated form of GroEL could not suppress any of the alleles, although a low level of integrative suppression was seen in all cases. One of the alleles tested, *dnaA167*, did show greater temperature resistance when transformed at 40°C with pHC18 rather than pVH1, but true suppression was not seen at 42°C. *DnaA167* is interesting as it is the only *dnaA* mutant suppressible by overexpression of *groE* that is not consequently cold sensitive, although the reasons for this remain obscure (Table 3.5.5).

These results were completely unexpected and cast serious doubt over the validity of conclusions drawn from previous observations. This problem had to be addressed; pJM32's ability to suppress the *dnaA46* mutation, as described by March, was therefore reinvestigated.

Table 3.5.4 Suppression of *dnaA46* (with *recA56*) by *groEL*$^+$ and *groEL*$_{tr}$.

Strain	Plasmid	Colony size		Transformation efficiency/μg DNA	
		30°C	40°C	30°C	40°C
MM22 (*dnaA46 recA56*)	pVH1	++	-	9.6×10^3	0
	pIR88 (*groEL*$^+$)	+[a]	++	5.2×10^2	1.6×10^4
	pHC18 (*groEL*$_{tr}$)	++	-	8.8×10^3	0
	pJF118	++	-	2.1×10^4	0
	pGTIR88 (*groEL*$^+$)	+[a]	++	8.3×10^2	1.8×10^4
	pGTHC18 (*groEL*$_{tr}$)	++	-	1.9×10^4	0

See Table 3.5.2 for experimental details.
++, well formed colonies; -, no colonies visible.
a. Cold sensitive combination.

Table 3.5.5 Suppression of a number of *dnaA* alleles by *groEL*$^+$ and *groEL*$_{tr}$.

Strain	Allele	Plasmid	Colony Growth 30°C	40°C	42°C
MM38	*dnaA*$^+$	pVH1	+++	+++	+++
		pIR88	+++	+++	+++
		pHC18	+++	+++	+++
MM181	*dnaA167*	pVH1	+++	+	-
		pIR88	+++	+++	++
		pHC18	+++	+++	-
MM182	*dnaA5*	pVH1	++	-	-
		pIR88	+	+	+
		pHC18	++	-	-
MM183	*dnaA204*[a]	pVH1	+++	-	-
		pIR88	++	-	-
		pHC18	++	-	-
MM184	*dnaA508*	pVH1	+++	-	-
		pIR88	++	-	-
		pHC18	++	-	-
MM185	*dnaA46*	pVH1	++	-	-
		pIR88	+	++	+++
		pHC18	++	-	-
MM186	*dnaA203*[a]	pVH1	++	-	-
		pIR88	++	-	-
		pHC18	++	-	-
MM187	*dnaA602*[b]	pVH1	++	-	-
		pIR88	+	++	++
		pHC18	++	-	-
MM188	*dnaA601*[b]	pVH1	++	-	-
		pIR88	+	++	++
		pHC18	++	-	-
MM189	*dnaA604*	pVH1	++	-	-
		pIR88	-	++	++
		pHC18	++	-	-

Strains were transformed with the given plasmids at suitable temperatures and the resulting transformants plated out onto L-agar at 30, 40 and 42°C.
Plates were incubated overnight and then examined for colony growth.
+++, well formed colonies; -, no colonies visible.
In the case of pHC18 at 40/42°C, '-' takes into account the colony formation at low frequency owing to integrative suppression seen with all the *dnaA* mutations tested.
a,b Recently it has been shown that these pairs of alleles carry the same mutations (Hansen *et al.*, 1992).

3.6 pJM32 does not suppress *dnaA* mutations via a GroE protein mechanism

The finding that the multicopy plasmids described above, which carry *groES* and the truncated version of the *groEL* gene, can only suppress temperature-sensitive *dnaA* mutants via an integrative mechanism implies that the originally observed suppression by pJM32 was likely to have occurred in the same manner. If this were the case then pJM32 suppression should also be *recA* dependent. Conversely, if the GroEL'–fusion protein product of pJM32 were mediating true $dnaA^{ts}$ suppression, then suppression should still occur in a *recA* background. In order to test this an attempt was made to transform a *dnaA46*, *recA56* strain with pJM32, but problems were encountered with this approach. Both the *dnaA46*, *recA56* strains in our collection (MM21 and MM22) are exceptionally sick cell lines. MM22 has been shown to produce anucleate cells at a rate of almost 1 in 4 at the permissive temperature of 30°C (S. Addinall, personal communication). Suppression of *dnaA46*, *recA56* strains by multicopy plasmids carrying the intact *groE* operon occurs at 40°C and not at 42°C unlike the isogenic *dnaA46*, $recA^+$ strain. Transformation efficiencies of these strains tend to be extremely poor, even with the most easily handled plasmids, and thus it was not entirely unexpected that the highly unstable and difficult to manage pJM32 could not be transformed into MM22, and after many unsuccessful attempts another strategy was sought.

Advantage was taken of the fact that pJM32 carries the ColE I origin of transfer region, *oriT*, inherited from pBR325. This meant that pJM32 should be transferable between strains by conjugation. To do this, the original *dnaA46* strain suppressed by pJM32, MM19(pJM32), was used as a conjugational donor. As a control MM19 was transformed with pIR2010, which is an Amp^R, Tet^S, Chl^S, $oriT^+$ derivative of pBR325. Both of these MM19 derivatives were transformed with pAC44 (kindly donated by C. Boyd) which is Chl^R, $oriT^-$ and carries *mob* genes, which are necessary for the mobilisation of $oriT^+$ plasmids during conjugation. These cells were then mixed with the strain CB71-18 which carries a transferable F', and with MM21 (*dnaA46*, *recA56*, Tet^R). In this triparental mating, the F' from

CB71-18 enters the MM19 strains, which in turn transfer the F' and the mobilized *oriT*$^+$ plasmids to MM21. The desired exconjugants can easily be selected by antibiotics, in this case ampicillin and tetracycline. Plasmid transfer by this method is highly efficient, and in the control experiment pIR2010 was found to easily transfer into MM21. However pJM32 transfer could not be detected at all (Table 3.6.1). This is consistent with the idea that pJM32 is integrated into the chromosome, rather than free to be transferred, and is suppressing the *dnaA* mutation in MM19 integratively. Unfortunately neither of the *groEL*$_{tr}$-containing plasmids that had been made (pHC18, pGTHC18) contained origins of transfer and so this experiment could not be performed with these plasmids.

Table 3.6.1 Triparental mating to transfer pJM32 to MM21.

Donor strain	Transferred plasmid	Recipient strain	No. of Tet^R/Amp^R Colonies (per unit of culture)	
			30°C	40°C
MM19 pAC44 (Chl^R)	pIR2010 (Amp^R)	MM21 (*dnaA46*, *recA*,Tet^R)	930	0
	pJM32 (Amp^R)		0	0

See text for experimental details.

Further evidence of pJM32's lack of GroE-dependent *dnaA* suppression was obtained using a plasmid constructed by Ian Oliver in this laboratory. The whole *groES*$^+$*EL'*–fusion insert from pJM32 was recloned into the Kan^R, ColD plasmid pVH1 to produce plasmid pIR89. Unlike pJM32, pIR89 was quite stable and gave good yields of plasmid DNA. When transformed into MM19 a small background suppression was seen (again most probably owing to chromosomal integration of the plasmid), but in MM22 no suppression was seen (Table 3.6.2).

Table 3.6.2 Suppression of *dnaA46* (with *recA56*) by *groEL*$^+$ and *GroEL'*–fusion.

Strain	Plasmid	Colony size		Transformation efficiency/μg DNA	
		30°C	40°C	30°C	40°C
MM22 (*dnaA46 recA56*)	pVH1	++	-	9.6×10^3	0
	pIR88 (*groEL*$^+$)	+[a]	++	4.9×10^2	1.1×10^4
	pIR89 (*groEL'*–fusion)	++	-	9.8×10^3	0

See Table 3.52 for experimental details.
+++, well formed colonies; -, no colonies visible
a. Cold sensitive combination

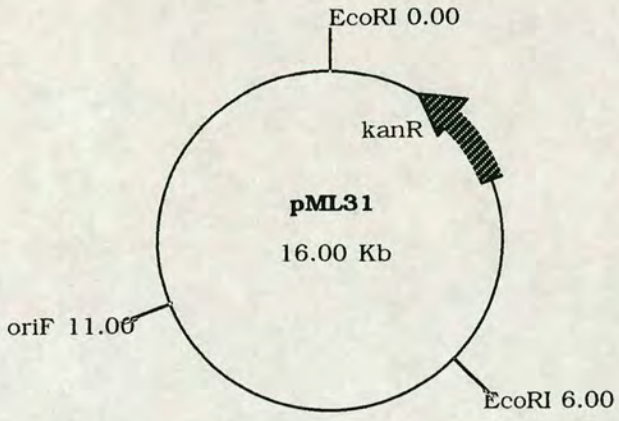
This confirmed that the protein products produced by pJM32 are in no way involved with suppression of *dnaA* mutations, as was proposed by March (1988). Absolute proof of chromosomal integration of pJM32 (and pHC18) in *dnaA46* strains could have been gained by Southern blotting the chromosomal DNA from such strains. However, this has not been done because it was felt that this was becoming very much a side issue in this project, and the other evidence for the lack of *dnaA46* suppression by the *groEL'*–fusion encoded by pJM32 (and $groEL_{tr}$ of pHC18) was quite compelling.

Although the initial conclusions regarding the role of pJM32 in *dnaA*^{ts} suppression were incorrect, they did lead to the unexpected finding that the $GroEL_{tr}$ protein, lacking its highly conserved GM tail, could still complement *groEL*^{ts} mutations; it was this observation that was to be further explored.

3.7 Does the ability of *groEL*$_{tr}$ to complement *groEL*^{ts} depend on its copy number?

Since it had been expected the GM tail of GroEL was essential, for the reasons described above, it was a surprise to find that *groEL*$_{tr}$ could complement all the *groEL*^{ts} strains in our collection. As *groEL*$_{tr}$ had so far been produced only from high-copy-number plasmids it was possible that the observed complementation could be caused by the high levels of truncated protein present in these cells. If GroEL$_{tr}$ were less active or less efficient in comparison with its wild-type counterpart, then the effects of this could be compensated for by elevated protein levels. SDS–PAGE protein analysis of cells carrying the GroEL$_{tr}$ on pHC18 showed that it is highly overproduced, being indistinguishable from the level of wild-type protein produced from pIR88 (see Figure 3.3.6). This also suggests that the truncated protein has a similar stability in the cell as does the wild-type. It was possible, however, that although the GroEL$_{tr}$ polypeptides are being efficiently produced they are not being as efficiently assembled into the tetradecameric ring structure as the wild-type protein. Again this could be masked by overexpression. If there were an equilibrium between GroEL monomers and tetradecamers within the cell then overexpression could shift the equilibrium towards the formation of the tetradecamers. To address the question of copy-number effects it was decided to clone the *groE* operons from pIR88 and pHC18 into a low-copy-number plasmid vector. The vector chosen was a Kan^R mini-F plasmid called pML31 (Figure 3.7.1).

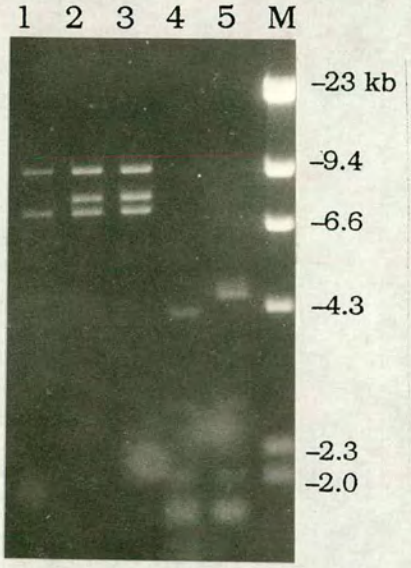
Figure 3.7.1



pML31, a 16 kb mini-F plasmid carrying a kanamycin-resistance gene. The plasmid has a copy number of 1–2 per cell (Lovett and Helinski, 1976).

This plasmid was originally isolated by restricting F DNA with *Eco*RI and ligating the products together with an *Eco*RI DNA fragment which conferred kanamycin resistance. pML31 was the smallest self-replicating Kan^R plasmid obtained (Lovett and Helinski, 1976). It consists of 16 kb of DNA, of which 10 kb is F-derived and contains two *Eco*RI restriction sites; its copy number is 1–2 per cell. For cloning into pML31 the plasmid DNA was partially digested with *Eco*RI. This DNA was mixed with the 8.1 kb *Eco*RI fragments from either pIR88 or pHC18; these were ligated together in the usual way. The ligation mixes were transformed into NL302, a *recA groES*^{ts} strain, and selection made for Kan^R colonies at the restrictive temperature of 42°C. This selected for recombinant plasmids carrying a wild-type *groES* gene, present on the 8.1 kb fragments. Plasmid DNA was extracted from transformants and the correct constructs were verified by restriction analysis of the DNA (Figure 3.7.2). The pML31-based plasmid derived from pIR88 was named pIRF1 (GroEL$^+$), and the one from pHC18 was named pHCF3 (GroEL$_{tr}$) (Figure 3.7.3).

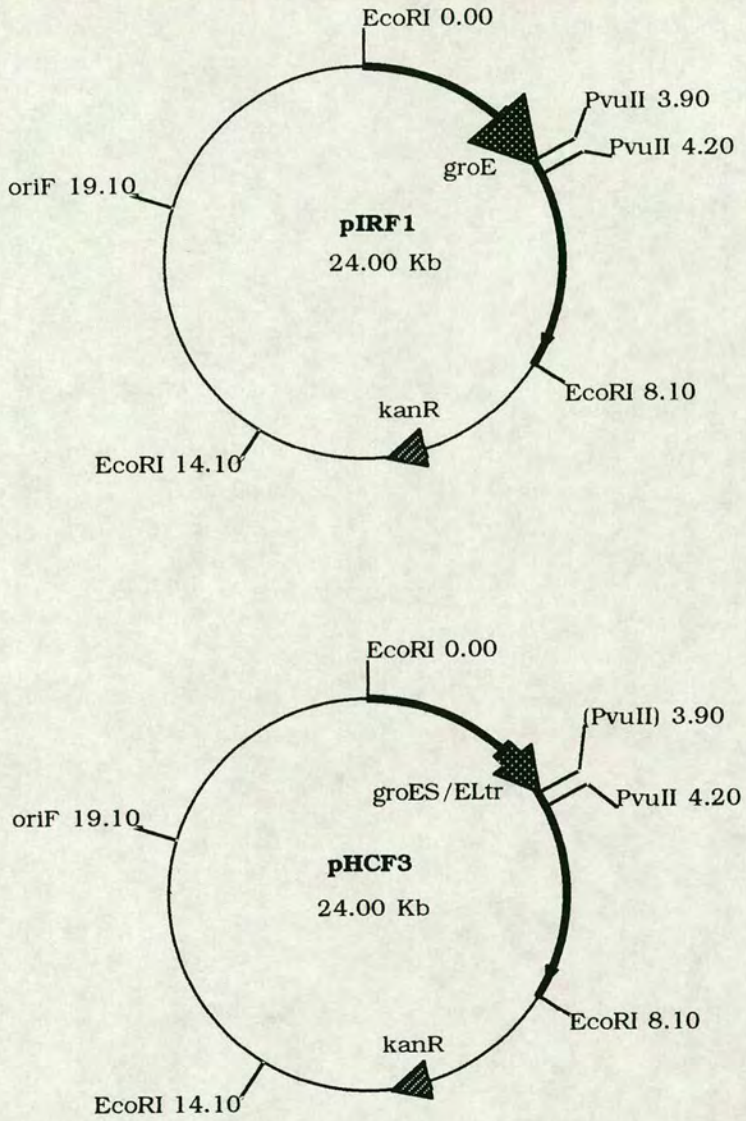
Figure 3.7.2 Restriction of pML31, pIRF1 and pHCF3.



1	pML31 *Eco*RI
2	pIRF1 *Eco*RI
3	pHCF3 *Eco*RI
4	pIRF1 *Pvu*II
5	pHCF3 *Pvu*II
M	λ *Hin*dIII markers

Lane 1 shows the 6 and 11 kb *Eco*RI fragments of pML31. Lanes 2 and 3 show the 8.1 kb *Eco*RI fragments from pIR88 and pHC18, respectively, cloned into pML31. Lanes 4 and 5 show the 4.2 and 4.5 kb fragments generated by *Pvu*II restriction with the size increase in pHCF3 owing to insertion of the Universal Translation Terminator into the *Pvu*II site just upstream of the GM motif encoding sequence of *groEL*.

Figure 3.7.3



pIRF1 and **pHCF3** were constructed by ligating the 8.1 kb *Eco*RI DNA fragments encoding the *groE* genes from pIR88 and pHC18, respectively, into pML31 partially digested with *Eco*RI.
(PvuII) denotes the *Pvu*II site lost owing to insertion of the Universal Translation Terminator and thus encoding GroEL**tr**.

These constructs were transformed into NL441 (*groEL*^{ts}), where it was found that the mutation could be complemented for growth at

42°C by both pIRF1 and pHCF3. Both plasmids also complemented NL441 for the maintenance of λ and T4/5 phages (Table 3.7.1).
It was also found that neither plasmid could suppress the *dnaA46* mutation in MM22. This lack of suppression was expected even for the *groE*$^+$ plasmid pIRF1, since it has been estimated that at least six copies of the *groE* genes are required for this suppression to occur (Fayet *et al.*, 1986).

These results demonstrate that the ability of a GM-minus form of *groEL* to complement *groEL*^{ts} mutations is not dependent on overexpression of the protein as complementation occured with only a single copy of the *groEL*$_{tr}$ gene present in the cell.

Table 3.7.1 Complementation of *groEL44* / suppression of *dnaA46* with *groEL*$^+$ and *groEL*$_{tr}$ from low copy-number plasmids.

Strain	Plasmid	Colony Size 30°C	42°C
NL441 (*groEL44*)	pML31	++	-
	pIRF1 (*groEL*$^+$)	++	+++
	pHCF3 (*groEL*$_{tr}$)	++	+++
		30°C	**40°C**
MM22 (*dnaA46*)	pML31	++	-
	pIRF1 (*groEL*$^+$)	++	-
	pHCF3 (*groEL*$_{tr}$)	++	-

Strains were transformed with the given plasmids and streaked out on L-agar with kanamycin at the stated temperatures and incubated overnight. Plates were then examined for growth.
+++, well formed colonies; -, no colonies visible.

3.8 Attempts to suppress other mutations with *groEL*$_{tr}$

GroEL mutations have been shown to suppress other temperature-sensitive mutations in *E. coli*. These include suppression of *ssb1* and *ssb113* (single-stranded DNA-binding protein) by *groEL441* and *groEL46*. In addition an *rpoH* deletion mutant (the RNA polymerase heat-shock transcription factor σ-32) can be suppressed by overexpression of the *groE* genes. In order to identify differences in the phenotypes conferred by GroEL$^+$ and GroEL$_{tr}$ it was decided to attempt to find out whether overexpression of GroEL$_{tr}$ could, in conjunction with GroES, suppress any of these mutations.

ssb

Plasmids pIR88, pHC18 and pVH1 were transformed into RM121 (*ssb1*) and RM139 (*ssb113*), at 30 and 42°C. The cells transformed efficiently at the permissive temperature of 30°C, but at 42°C no transformants were obtained. Colonies were picked from the 30°C transformation plates and streaked out at 30 and 42°C. Again no colonies were seen to grow at the higher temperature. It could be seen that temperature-sensitive *ssb* mutants are not suppressed by overexpression of the wild-type GroE proteins, nor by overexpression of GroES and GroEL$_{tr}$ proteins. The finding that overexpression of wild-type GroE proteins does not suppress *ssb* mutations is consistent with previous observations (Ruben *et al.*, 1988; Laine and Meyer, 1992); we show here that using GroEL$_{tr}$ is also ineffective.

rpoH

Suppression of an *rpoH* deletion by overexpression of *groE* was demonstrated by Kusukawa and Yura (1988). The *rpoH* deletion strain can normally only survive at temperatures lower than 20°C; however concomitant overexpression of the *groE* operon allows growth up to 40°C. If the heat-shock protein DnaK is overexpressed along with the GroE proteins then the deletion strain can grow up to 42°C. An *rpoH* deletion strain was not available in our laboratory, but an *rpoH*-amber mutant with a temperature-sensitive tRNA suppressor was. This strain, OV32, was transformed with pIR88, pHC18 and pVH1 at 30 and 42°C. The strain transformed well at the lower temperature, but

no transformants were obtained at 42°C for any plasmid. Restreaking the 30°C transformants at 42°C did not result in visible growth at the higher temperature. When the strains were streaked out at the intermediate temperature of 40°C, the control strain carrying pVH1 grew as well as strains overproducing either the wild-type or truncated forms of GroEL, and so any advantage conferred to OV32 by *groE* was not apparent. Since overexpression of *groE* alone did not allow growth at 42°C of the original *rpoH* deletion strain, it may have been expected that suppression of an amber mutant would also not occur at 42°C. However, since *rpoH* deletion strains are so biologically fragile, and in comparison OV32 is a healthy strain, it could have been that *groE* suppression may have occurred at higher temperatures but was not seen. From these experiments it cannot be acertained whether the truncated form of the GroEL protein can allow suppression of temperature-sensitive *rpoH* mutations; attempting to suppress an *rpoH* deletion strain may be the only way to answer this question. Alternatively co-overexpression of *dnaK* from a multicopy plasmid along with *groE* should allow OV32 to grow at 42°C and would provide a system for testing $groEL_{tr}$.

3.9 Summary and Discussion

Work originally undertaken in this laboratory by Jenkins (1985) which showed that overexpression of the GroE proteins could suppress the temperature sensitivity of certain *dnaA* mutations, was continued by March (1988). He constructed a plasmid, pJM32, which carries the *groES*$^+$ gene and the first 495 codons of the *groEL* gene (out of a total 548) fused to an out-of-frame portion of the tetracycline-resistance gene of pBR325, and observed that this plasmid could not complement *groEL*^{ts} mutations, but could suppress *dnaA*^{ts} mutations despite a low copy number. It was postulated that because the GroEL'–fusion protein encoded by pJM32 had lost carboxyl-terminal sequences, it had become more efficient than the wild-type GroEL at interacting specifically with DnaA, although was unable to perform all the necessary functions of the wild-type GroEL protein.

Searches of the SWISSPROT data base demonstrated that the carboxyl-terminus of *E. coli* GroEL protein has an array of repeated glycine–methionine (GM) residues that are highly conserved among the HSP60 class of molecules. Attention was focused on this sequence and caused speculation that it might have a specific and important role in GroEL function. Interestingly the eukaryotic cytosolic chaperonin TCP-1 does not contain the GM motif. Similar GM repeats are found in some HSP70 molecules. HSP60- and HSP70-type molecules are believed to interact *in vivo*. Perhaps in some circumstances the HSP60/70 heat-shock apparatus requires the presence of a GM motif, normally supplied by HSP60, but in the case of TCP-1 supplied by HSP70.

The homologies and ubiquity of this amino acid sequence seemed to imply that this region of the GroEL protein would play some vital role in normal GroEL function but nothing is known about the possible role of such a motif. Steinert *et al.* (1991) suggested that glycine-rich domains can be organized into loops with concomitant stacking of the adjacent hydrophobic residues. The glycine-repeat loops between these stacks could create a flexible structure akin to a molecular spring. They also suggest that individual molecules could interact by way of these glycine loops to form arrays. This is true for structural proteins such as keratins and cytokeratins but in these

cases the glycine domains are considerably larger than the 13 amino acids of the GroEL GM motif.

Since pJM32 was a particularly difficult plasmid to work with, it was decided to clone a version of the *groEL* gene that encodes a GM-deficient protein. This was achieved by cloning an oligonucleotide with TAA STOP codons in all three reading frames into a *Pvu*II restriction site immediately upstream of the DNA encoding the GM tail of the protein, resulting in plasmids pHC18 and pGTHC18. Much to our surprise, these plasmids, expressing truncated GroEL protein (GroEL$_{tr}$), were able to complement all the *groEL*^{ts} mutations in our collection for both high-temperature growth and the maintenance of phages λ, T4 and T5, but did not suppress *dnaA*^{ts} mutations at high temperature. Strains containing them did not exhibit the cold sensitivity associated with GroE-mediated *dnaA*^{ts} suppression. One of the *dnaA*^{ts} alleles tested, *dnaA167*, did show a degree of temperature resistance when expressing GroEL$_{tr}$, possibly suggesting that the GroEL$_{tr}$ was having some effect on the mutant DnaA protein but less than that of the wild-type protein. *DnaA167* strains were the only *dnaA* mutants that were not cold sensitive in the presence of overexpressed GroE proteins and so maybe it is 'easier' to suppress than the other mutations. The possibility for a reduced activity of GroEL$_{tr}$ compared to the wild-type protein made me wonder if the complementation of *groEL*^{ts} mutants by *groEL*$_{tr}$ could be gene dosage dependent but even in a single copy-number mini-F'-based vector the truncated form of *groEL* could complement *groEL*^{ts} mutations for high temperature and phage growth indicating that complementation by this form of the gene was not dependent on a high gene dosage. It must also be stated that the *groEL*$_{tr}$-complemented mutants showed no apparent disadvantages when compared to cells complemented by the wild-type form of *groEL*. These results, together with observations that pHC18 could suppress *dnaA*^{ts} via an integrative (GroE protein independent) mechanism cast doubt upon the validity of March's original interpretation of his observations. I subsequently showed that pJM32 could not suppress *dnaA*^{ts} mutations in a *recA* strain background, and that the GroEL'–fusion protein did not suppress *dnaA*^{ts} mutations when recloned into a more manageable vector. This suggested that pJM32 was actually suppressing the *dnaA*^{ts} mutations

by integration of the plasmid replicon into the chromosome, presumably using the *groE*-containing DNA insert for homologous recombination. This would bypass the need for the DnaA protein and in this way allow chromosomal DNA replication in $dnaA^{ts}$ mutants at normally non-permissive temperatures.

In addition, attempts were made to determine whether overexpression of $groEL_{tr}$ could suppress other temperature-sensitive mutations (*ssb* and *rpoH*), but no genetic suppression was seen.

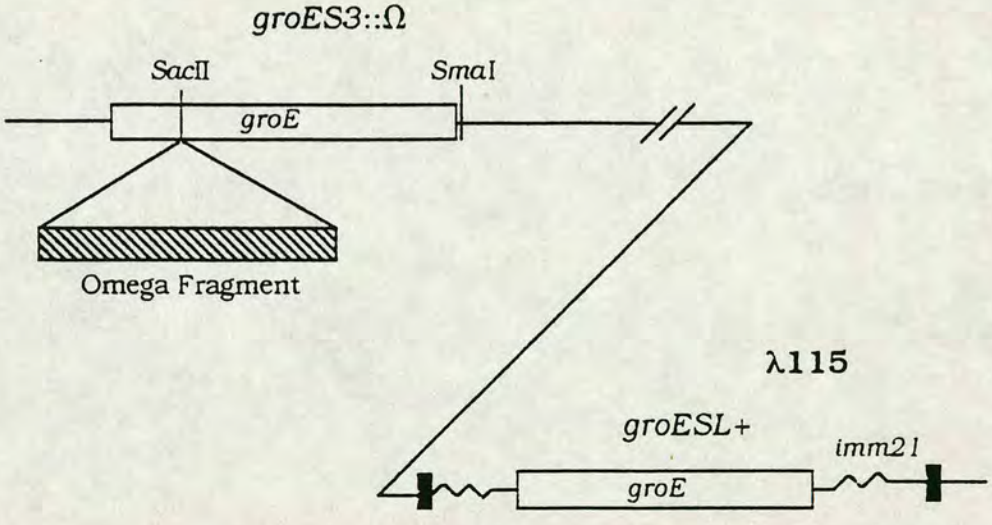
CHAPTER 4

CONSTRUCTION AND COMPLEMENTATION OF *GROE*-NULL STRAINS WITH $GROEL_{TR}$

4.1 Introduction

The finding that *E. coli* has an absolute requirement for both the GroEL and GroES proteins at all growth temperatures was first reported by Fayet *et al.* (1989). In these experiments a strain was constructed (OF216) that carries a DNA insertion in the chromosomal *groES* gene. The inserted DNA is called the omega fragment (Ω) and carries genes encoding resistance to the antibiotics streptomycin and spectinomycin (Str^R/Spc^R) (Prentki and Krisch, 1984). The mutation was given the allele number *groES3*::Ω. The Ω DNA contains strong transcriptional terminators derived from bacteriophage T4 at its ends, which have a polar effect on transcription. As a result *groEL* transcripts are not synthesized and so *groES3*::Ω strains are also *groEL*$^-$. OF216 is lysogenic for the λ phage (λ115), which carries the wild-type *groE* operon and so permits growth (Figure 4.4.1).

Figure 4.4.1 Genetic arrangement of strain OF216.



Genetic arrangement of strain OF216 showing the Ω fragment located in the *Sac*II site in the chromosomal copy of *groES*. The mutation is complemented by the lysogenic phage λ115 inserted in *attλ*, which carries intact *groE* genes.

It was shown that the antibiotic resistances of *groES3*::Ω could be transferred to another strain via phage P1-mediated transduction only if the recipient strain carried either λ115, or a plasmid encoding the

groE genes. It was also found that neither of the *groE* genes could be deleted individually. This is true over a wide temperature range (18–42°C) indicating that the GroE proteins are vital for normal cellular function throughout this range of temperatures, and not just at the higher temperatures usually associated with the heat-shock response.

Strain OF216 was used in this study to test the ability of modified GroE proteins to complement the GroE-null insertion *groES3*::Ω.

4.2 Truncated *groEL* can complement *groES3*::Ω

The truncated form of GroEL is able to replace $GroEL^{ts}$. In order to exclude the possibility that this results from intergenic complementation, in which temperature-sensitive GroEL subunits combine with $GroEL_{tr}$ to generate heterologous GroEL tetradecamers that are active, it was decided to test whether $groEL_{tr}$ could complement the *groE*-null mutation *groES3*::Ω. OF216 was very kindly given by O. Fayet (Fayet *et al.*, 1989) and a P1 lysate prepared on it. The strain NM306 was used as a recipient for the P1 transduction. This strain is *purA* and requires adenine for growth. *PurA* is approximately 15% cotransducible with the *groE* operon. NM306 was transformed with the mini-F-based plasmids pML31, pIRF1 or pHCF3, which have been described previously. The rationale behind the experiment was that the P1 lysate from OF216 would be used to transduce all the plasmid-bearing NM306 derivatives from *purA* to $purA^+$, and that these resulting transductants would then be screened for coinheritance of the Str^R/Spc^R of the Ω fragment. Only those cells that carry functional *groE* genes *in trans* should be able to inherit the Ω fragment. This approach avoids the detection of cells which have become Str^R/Spc^R owing to recombination with the plasmid-borne *groE* genes since such cells will not be $purA^+$. The results from these experiments are shown in Table 4.2.1.

Table 4.2.1 Transduction of NM306 with P1 OF216 (*groES3*::Ω).

Recipient Strain	No. of $purA^+$ Progeny	No. of Str^R/Spc^R Progeny	Linkage (%)
NM306 pML31	141	0	0
NM306 pIRF1	168	25	15
NM306 pHCF3	171	24	14

Strains were transduced to $purA^+$ using a P1 lysate on OF216. Transductants were then patched onto plates +/– Str/Spc and incubated overnight at 30°C. Patch growth was then scored accordingly.

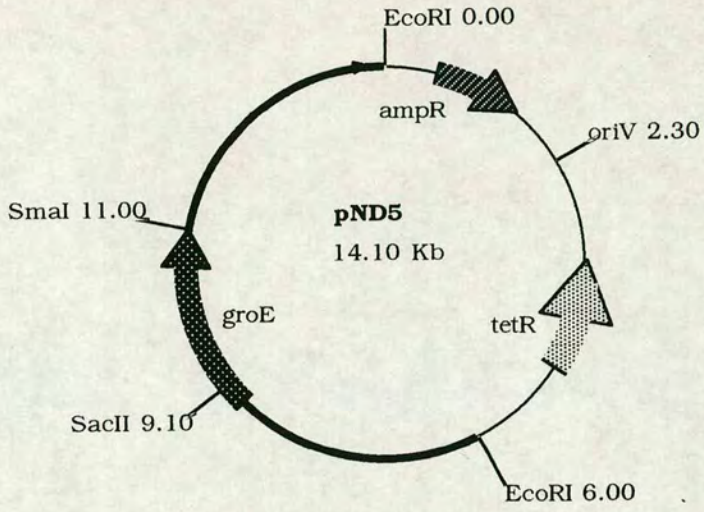
It can be seen that *purA*$^+$ transductants of NM306 cells carrying the *groE*$^-$ plasmid pML31 never show coinheritance of the Ω fragment, a result that agrees with the observations of Fayet *et al.* (1989). However, when the strain carried *groES* plus either the wild-type *groEL* gene (pIRF1), or the truncated version (pHCF3) similar cotransduction frequencies of *purA*$^+$ gene and Ω are seen. This repeatable result suggested that cells expressing only *groEL*$_{tr}$ are fully viable. This was surprising because, as discussed in *Chapter 3*, the high degree of sequence homology seen in the carboxyl-termini regions of virtually all the GroEL-like heat-shock proteins characterized to date suggest that it was likely to be an important motif; it could, however, be the case that the tail of the protein is only required for some specific non-vital role in GroEL function. This point will be examined in detail later.

4.3 Construction of a *groE*-deletion strain

A possible problem with the above experiments was the fact that *groES3*::Ω retains *groE* DNA. This allele is not a deletion of the *groE* locus but is actually an insertion mutant in which the Ω fragment DNA resides in the *Sac*II restriction site of the *groES* gene (see Figure 4.4.1). The *groEL* gene in this construction has an uninterrupted wild-type sequence, but since *groEL* does not have its own promoter and given that the Ω fragment has a severe polar effect on downstream transcription (owing to the presence of transcriptional terminators at its ends) the construction is effectively *groEL*$^-$. However, this does mean that the *groEL* DNA is potentially available for events such as homologous recombination, and the possibility of transcriptional read-through from the powerful promoters of the *groE* operon upstream of the Ω fragment could not be discounted. It was therefore thought possible that some GroEL protein could be produced from this construction, perhaps not enough to allow survival of the cell, but sufficient so that, in combination with the truncated form of GroEL, the observed complementation could result. It was decided that these possibilities should be excluded by constructing a true *groE*-deletion strain. The approach taken was to make the deletion of the *groE* genes on a plasmid and then to homologously recombine the plasmid insert into the chromosome of a cell while supplying the *groE* genes *in trans* from a recombinant plasmid or lysogenic phage.

Creating the deletion on a plasmid involved the use of pND5 which carries the 8.1 kb *Eco*RI chromosomal DNA fragment with the 2.2 kb *groE* operon near its centre. The Fayet construction utilized a unique *Sac*II restriction site located within the *groES* gene to insert the Ω fragment DNA. I decided to also use this site as the 5' junction of the deletion, and as the 3' end, the *Sma*I site located just downstream of the *groEL*-coding sequence. Both the *Sac*II and *Sma*I recognition sites are unique within pND5 (Figure 4.3.1).

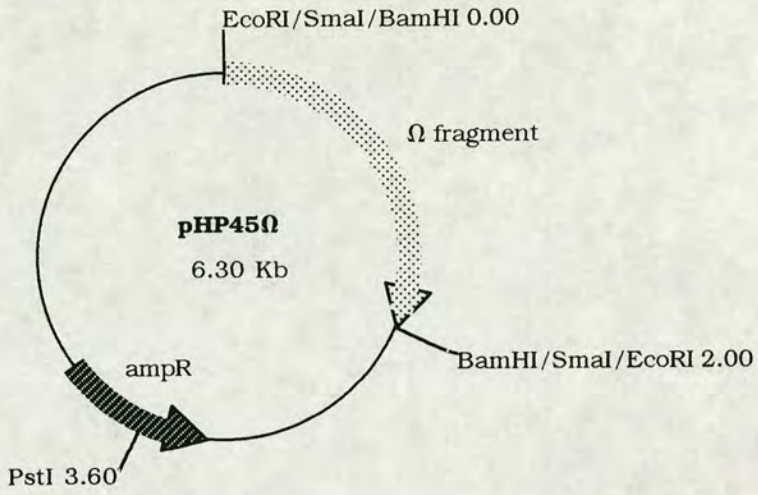
Figure 4.3.1



Restriction map of **pND5** showing the positions of the *Sac*II and *Sma*I sites intended for use in construction of a *groE*-deletion strain.

As in the Fayet construction the Ω fragment encoding both streptomycin and spectinomycin resistances would be used, but in this case it would replace (rather than insertionally inactivate) the *groE* genes. The Ω fragment can be efficiently excised from plasmid pHP45Ω (Prentki and Krisch, 1984) using several restriction endonucleases including *Sma*I (Figure 4.3.2).

Figure 4.3.2

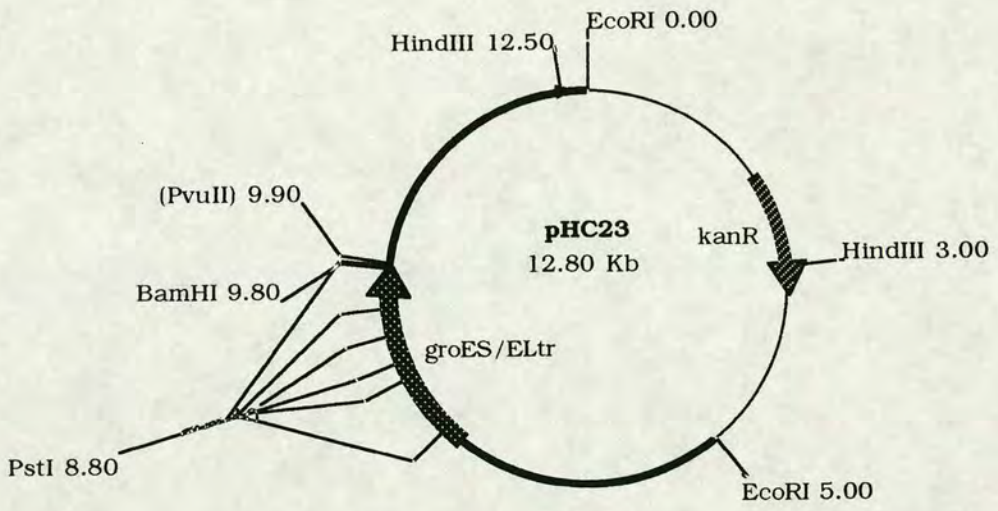


Restriction map of **pHP45Ω** (Prentki and Krisch, 1984). The omega fragment (Ω) can be excised using *Eco*RI, *Bam*HI , *Sma*I and *Hin*dIII (not shown).

*Sma*I restriction produces blunt-ended DNA molecules, which can be ligated to other blunt-ended molecules. Unfortunately *Sac*II digestion results in DNA molecules with 3'-overhanging ends. This was to produce problems since 3'-overhanging DNA molecules cannot be blunted as easily as 5'-overhanging ends, which can be 'filled-in' using the DNA polymerase activity of Klenow enzyme. Attempts were made to blunt the *Sac*II-digested DNA using both the exonuclease activity of T4 DNA polymerase and the single-stranded DNA exonuclease activity of mung bean nuclease. Neither of these approaches was successful. It was felt that it was probably the blunting of the *Sac*II site that was causing the problems, but ligating blunt-ended DNA molecules can also be rather inefficient. Instead of persevering with this approach a new strategy was devised. The *groE* operon contains six *Pst*I restriction sites throughout its length, which could be used for constructing the deletion. *Pst*I digestion also results in DNA molecules with overhanging 3'-ends but these can be efficiently ligated to *Bam*HI-generated 5'-overhanging ends by using a *Pst*I–*Bam*HI single-stranded adaptor oligonucleotide (New England Biolabs; Product no. 1110). The Ω fragment can be excised using *Bam*HI and so this

approach would not require post-restriction enzymatic modification nor inefficient blunt-ended ligation. However, the resulting construction would still contain the region of the *groEL* gene encoding the GM tail of the protein, recombination with which could possibly interfere with results in a similar way to the Fayet construction. This problem was overcome by using pHC23, which had been isolated at the same time as pHC18. pHC23 encodes a truncated version of GroEL identical to that produced by pHC18, but in pHC23 the Universal Translation Terminator oligonucleotide is immediately followed by a 300 bp *Pvu*II deletion generated during the cloning and thus the GM-encoding DNA is missing. For this reason pHC23 was used for the construction of the *groE* deletion; the only *Pst*I restriction sites in pHC23 are the ones located in the *groE* region (Figure 4.3.3).

Figure 4.3.3

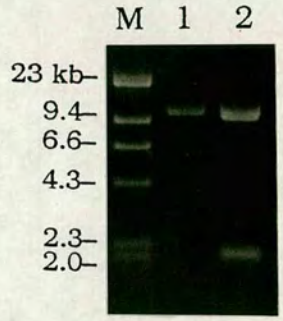


Restriction map of **pHC23**. The positions of the *Pst*I sites used in the construction of a *groE*-deletion strain are shown. The (PvuII) site was lost during the construction of pHC23.

The *Pst*I-restricted pHC23 DNA was ligated to pHP45Ω restricted with *Bam*HI in the presence of the *Pst*I–*Bam*HI adaptor oligonucleotide and the ligation mix was transformed into DH1 selecting for kanamycin (pHC23) and streptomycin/spectinomycin (Ω fragment) antibiotic resistances. Plasmid DNA from the resulting transformants was

analysed by restriction (Figure 4.3.4), and shown to be unable to complement either *groES* (NL302) or *groEL* (NL441) temperature-sensitive mutants. The correct constructon was designated pHCΩ1 (Figure 4.3.5).

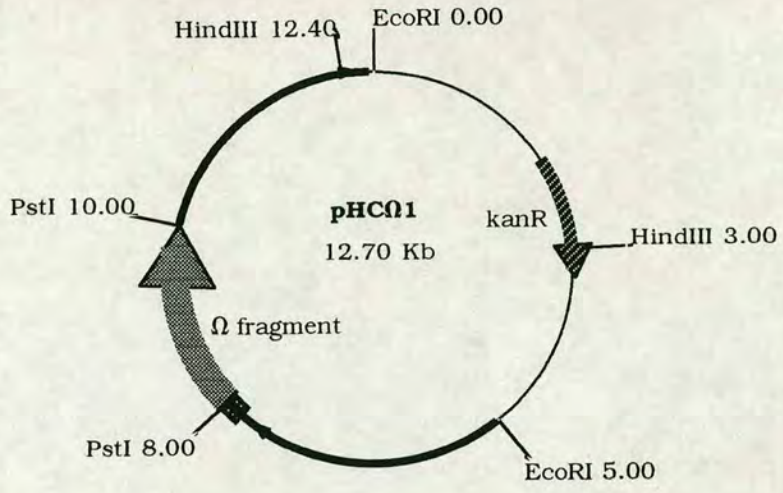
Figure 4.3.4 Restriction of pHC23 and pHCΩ1.



M	λ *Hin*dIII markers
1	pHC23 PstI
2	pHCΩ1 PstI

Plasmids pHC23 and pHCΩ1 were restricted with PstI, electrophoresed through a 0.8% agarose gel and stained with ethidium bromide. Note the 2.0 kb Ω fragment excised by PstI digestion of pHCΩ1.

Figure 4.3.5



pHCΩ1 was made by ligating the Str^R/Spc^R resistance encoding Ω fragment from pHP45Ω, restricted with *Bam*HI, into the flanking *Pst*I sites in pHC23. In order to join together the *Bam*HI and *Pst*I ends a suitable single-stranded adaptor oligonucleotide was used. This resulted in loss of the *Bam*HI sites, but retention of the *Pst*I sites flanking the Ω fragment. pHCΩ1 contains about 90 bp of *groES*, 70 bp of *groEL* and no GM tail-encoding DNA at all.

Interestingly a *groES*$^+$,*EL*Δ plasmid was also isolated (pHCΩ2), the result of a partial *Pst*I digestion of pHC23. This plasmid complements the *groES* mutation of NL302 well, whereas another commonly used *groES*$^+$*EL*$^-$ plasmid, pS4, does not (in my hands). pS4 also causes cells to grow poorly (cited in Zeilstra-Ryalls, 1991b) but pHCΩ2 shows no such debilitating qualities. It is possible that the GroES protein encoded by pS4 has mutated in a way that interferes with normal cellular function and that wild-type GroES can be tolerated at high levels without such problems. Of course it is possible that the plasmid copy number of pS4 (ColE-derived replicon) is much greater than pHCΩ2 (ColD replicon) and that the resulting higher levels of GroES cause the growth problems. This seems unlikely since the copy number of all the pVH1-derived plasmids used here appears to be high. pHCΩ2 was not used in the rest of this project but no doubt it will be of use in the future.

The next stage in constructing the *groE* deletion strain was to recombine the pHCΩ1 *Eco*RI fragment carrying the *groE*Δ::Ω allele

onto the chromosome at the *groE* locus. The initial approach involved transformation of linearized DNA fragments into a strain carrying a mutant *recD* gene (Russell *et al.*, 1989). The RecD protein is a subunit of the RecBCD nuclease in *E. coli* and linear DNA fragments are far more resistant to degradation in *recD* strains. Of course linear DNA fragments lacking replication origins cannot replicate in *E. coli* and thus maintenance of a genetic marker encoded by a linear DNA molecule can only occur by recombination into the chromosome (or some other replication-competent unit). *RecD* strains are not reduced in their capacity for homologous recombination. In this case the *recD* strain DL307 was used. Since *groE* genes are essential for cellular viability a deletion of the *groE* operon can only occur if another copy of the *groE* genes are supplied *in trans*, and so DL307 was lysogenized with λ*sidA* (*groE*$^+$). pHCΩ1 was restricted with *Eco*RI, the DNA extracted with phenol/chloroform, precipitated with ethanol, resuspended in TE and then cut a second time with the same enzyme in order to maximize the level of restriction. *Eco*RI digestion of pHCΩ1 results in two DNA fragments (see Figure 4.3.5). The fragment that encodes the *groE* deleted-Ω DNA does not carry a replication origin and so autonomous replication is impossible. Ten micrograms of this DNA was used to transform DL307(λ*sidA*). Transformants were selected using L-agar plates containing streptomycin/spectinomycin. It was found that the transformation efficiency was reduced 6×10^{-5}-fold when comparing *Eco*RI restricted and unrestricted pHCΩ1 DNA, indicating that the restriction of the DNA had been efficient. Transformants in which linear DNA had been recombined with the chromosome should have lost the kanamycin resistance encoded by the unwanted *Eco*RI fragment of pHCΩ1 (Table 4.3.1).

Table 4.3.1 Transformation of DL307 λ*sidA* with pHCΩ1 (restricted/ unrestricted).

Recipient Strain	No. of Str^R/Spc^R Colonies per 10 μg DNA pHCΩ1		No. of $Kan^S/Str^R/Spc^R$ Colonies per 10 μg DNA pHCΩ1
	unrestricted	*Eco*RI restricted	*Eco*RI restricted
DL307 λ*sidA* (*recD*)	$1.6x10^7$	27	5

DL307 λ*sidA* was transformed with 10 μg of either *Eco*RI-restricted or unrestricted pHCΩ1 DNA and plated onto L-agar containing streptomycin and spectinomycin and incubated overnight at 37°C. (In the case of the unresricted plasmid DNA dilutions of the transformations were plated out.) Resulting colonies were counted and the transformation efficiency calculated.
For the restricted DNA the resulting colonies were patched onto plates +/– kanamycin at 37°C and incubated overnight. The patches were scored for growth accordingly.

From two separate experiments a total of 27 Str^R/Spc^R colonies were produced of which only five were sensitive to kanamycin. It was assumed that the $Str^R/Spc^R/Kan^R$ colonies were carrying intact plasmids that had managed to survive *Eco*RI digestion without being cut, or alternatively had only been cut at one of the two *Eco*RI sites and thus the whole linearized plasmid had either integrated into the chromosome, or recircularized *in vivo*. Ten of the twenty two $Str^R/Spc^R/Kan^R$ clones were purified through several cycles in the absence of kanamycin selection and presence of Str/Spc selection, but Kan^R and Str^R/Spc^R were never found to segregate. The five $Str^R/Spc^R/Kan^S$ colonies were purified and tested for the presence of *groE* at the 94 minute chromosomal locus since it was equally likely that the *groE* genes of the lysogenized λ*sidA* had been replaced. The location of the insertions of pHCΩ1-derived DNA and confirmation of their *groE* deletion status was determined in the same experiment. P1 lysates were made on the five $Str^R/Spc^R/Kan^S$ derivatives of DL307(λ*sidA*) and these were used to transduce NM306 (*purA*) carrying either pML31 (*groE*$^-$) or pIRF1 (*groE*$^+$) to *purA*$^+$. *PurA*$^+$ clones should be produced in all cases. If the deletion of the *groE* genes at 92 minutes had been successful then 15% of the *purA*$^+$ clones should be Str^R/Spc^R when NM306 carries pIRF1 (*groE*$^+$), but no linkage between

purA and Str/Spc resistance should be seen in cells carrying pML31 (Table 4.3.2).

Table 4.3.2 Transduction of NM306 derivatives with P1 lysates from $Str^R/Spc^R/Kan^S$ progeny of DL307 transformed with pHCΩ1 *Eco*RI-restricted DNA.

P1 transduction donor Ssrain	Recipient strain	No. of colonies *purA*+	No. of colonies Str^R/Spc^R	Linkage (%)
1	NM306 pML31	89	0	0
	pIRF1	76	0	0
2	NM306 pML31	102	14	14
	pIRF1	123	17	14
3	NM306 pML31	45	0	0
	pIRF1	88	0	0
4	NM306 pML31	114	0	0
	pIRF1	78	0	0
5	NM306 pML31	93	12	13
	pIRF1	106	16	15

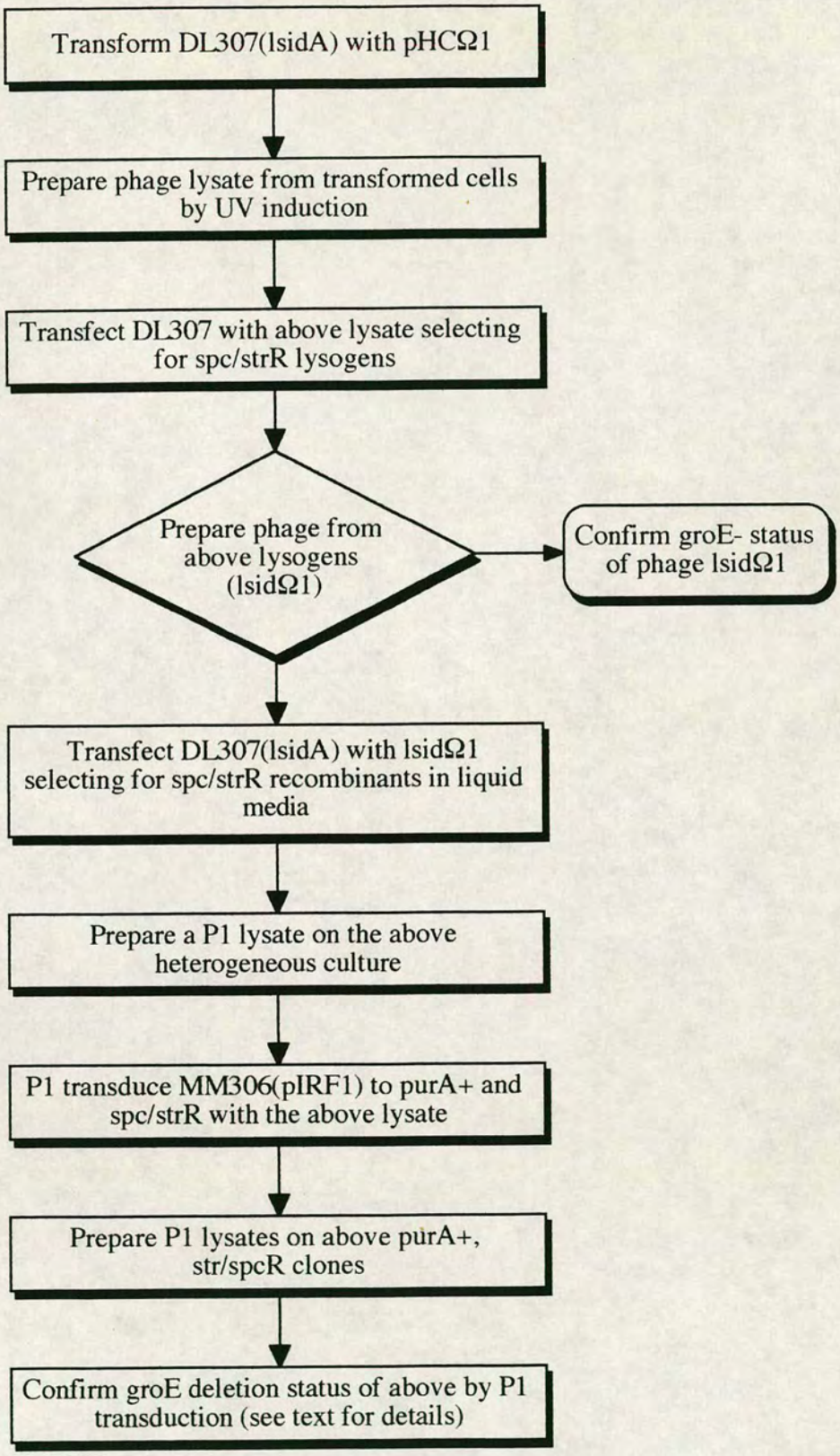
See Table 4.2.1 for experimental details.

Of the five strains analysed, three showed no linkage between *purA* and Str^R/Spc^R. The other two strains did show linkage at about 15% but this linkage was seen in NM306 with both pML31 and pIRF1. So in these cases a recombination event had occured in the correct region of the chromosome but this had failed to delete the *groE* genes. It is unclear what could have occured in the recombination to give this outcome, but in attempts to delete the *groEL* gene (leaving *groES* intact) using a comparable approach another research group have had similar results (P. Lund, personal communication). It is possible that there is a degree of homology between a part of the 8.1 kb *Eco*RI *groE* fragment and some other locus near the *groE*/*purA* region.

An even less successful approach was the one successfully employed by Winans *et al.* (1985) and Jasin and Schimmel (1984). Here strains with *recBC*, *sbcBC* mutations are transformed with linearized plasmid DNA. These strains cannot replicate plasmids and so maintenance of plasmid-borne genetic markers is caused by chromosomal integration. The *recBC*, *sbcBC* strain DL51, lysogenized with λ*sidA* (*groE*$^+$), was transformed with *Eco*RI-restricted pHCΩ1 on several occasions but no positive (Str^R/Spc^R/Kan^S) transformants were ever found.

Another approach to insertion of the *groE* deletion of pHCΩ1 was devised based on the method of Kulakauskas *et al.* (1991). This method employs phage λ to introduce mutated DNA fragments into the cell; this is much more efficient than DNA transformation. A strain carrying a plasmid in which the desired mutation/genetic marker is cloned is infected with a λ phage carrying the equivalent unmutated DNA. The resulting phage lysate will possess a minority of phages that have acquired the marker through homologous recombination with the plasmid. This lysate can now be used to infect another strain and recombinants can be found by selecting for the marker. In the original publication, λ phages belonging to the Kohara phage library were used (Kohara *et al.*, 1987). Kohara phages are useful for this purpose since they carry a hybrid attachment site and are effectively λ*att*-minus and are thus unable to lysogenize cells through normal means. Verification of the desired genetic rearrangement can be achieved by several methods (e.g. DNA sequencing, DNA hybridization studies, analysis of protein products). In this case a Kohara phage was not used since λ*sidA* seemed to be well suited to the task. A summary of this simple, but many stepped method is given in Figure 4.3.6..

Figure 4.3.6 Summary of the method used to construct the *groE*-deletion strain.



DL307(λ*sidA*) was transformed with pHCΩ1. The phage were induced by UV irradiation, which should also stimulate recombination. The resulting lysate was used to lysogenize DL307(λ$^-$) selecting for Str/Spc resistant clones. Four were induced with UV irradiation. Each of the resulting lysates lacked *groE*$^+$ phage since, unlike the parent phage λ*sidA*, they could not form plaques on *groE*^{ts} strains. One of these lysates (designated λ*sid*Ω1) was used in subsequent manipulations. λ*sid*Ω1 was used to infect DL307(λ*sidA*) and Str^R/Spc^R cells were selected in liquid medium. Str/Spc resistance could arise either by recombination between λ*sid*Ω1 and the chromosome, or more likely, since the target is larger (50 kb versus 6 kb), at the λ*sidA* locus. A P1 lysate was prepared on the mixture of Str^R/Spc^R cells and this lysate was used to transduce NM306 pIRF1 from *purA* to *purA*$^+$ selecting for Str^R/Spc^R at the same time. A total of four *purA*$^+$, Str^R/Spc^R colonies were produced and these were checked for the correct gene arrangement as before. P1 lysates were made on these clones and used to transduce NM306 carrying either pML31 or pIRF1 to *purA*$^+$ and these transductants tested for inheritance of the Str/Spc resistance of the Ω fragment. Of the four clones tested two showed linkage between *purA* and Str^R/Spc^R but only when *groE* genes were supplied *in trans* from pIRF1 (Table 4.3.3). The two clones that showed no linkage between *purA* and Str^R/Spc^R could have been λ*sid*Ω1 lysogens and P1 transductants since λ*sidA* and λ*sid*Ω1 would have been present in the P1 lysate. In retrospect, these could have been avoided by using a λ^R form of NM306 as the final transduction recipient.

Table 4.3.3 Transduction of NM306 derivatives with P1 lysates from *purA*$^+$, Str^R/Spc^R NM306 pIRF1 resulting from the Kulakaukas procedure.

P1 transduction donor strain	Recipient strain	No. of colonies *purA*$^+$	No. of colonies Str^R/Spc^R	Linkage (%)
1	NM306 pML31	101	0	0
	pIRF1	78	0	0
2	NM306 pML31	89	0	0
	pIRF1	113	18	16
3	NM306 pML31	130	0	0
	pIRF1	119	17	14
4	NM306 pML31	96	0	0
	pIRF	75	0	0

See Table 4.2.1 for experimental details.

It looked very likely that strains 2 and 3 above had been carrying the desired *groE* deletion and one of the P1 lysates giving this result (designated P1.2 ESLΔ) was used in later parts of this work.

4.4 Truncated GroEL can complement a *groE*-deletion strain

P1.2ESLΔ was used in the same way as the P1 lysate made on strain OF216 to transduce NM306 carrying either pML31 (*groE*$^-$), pIRF1 (*groE*$^+$) or pHCF3 (*groES*$^+$, EL_{tr}) to *purA*$^+$. *PurA*$^+$ transductants were screened for inheritance of the Str/Spc resistance from the *groE* replacing Ω-fragment. As before, cells carrying $GroEL_{tr}$ as their only form of GroEL protein seem to be just as viable under the conditions used and are produced at similar numbers to their GroEL$^+$ counterparts (Table 4.4.1).

Table 4.4.1 Transduction of NM306s with P1 ESLΔ::Ω.

Recipient strain	No. of *purA*$^+$ progeny	No. of Str^R/Spc^R progeny	Linkage (%)
NM306 pML31	238	0	0
NM306 pIRF1	249	37	15
NM306 pHCF3	282	45	16

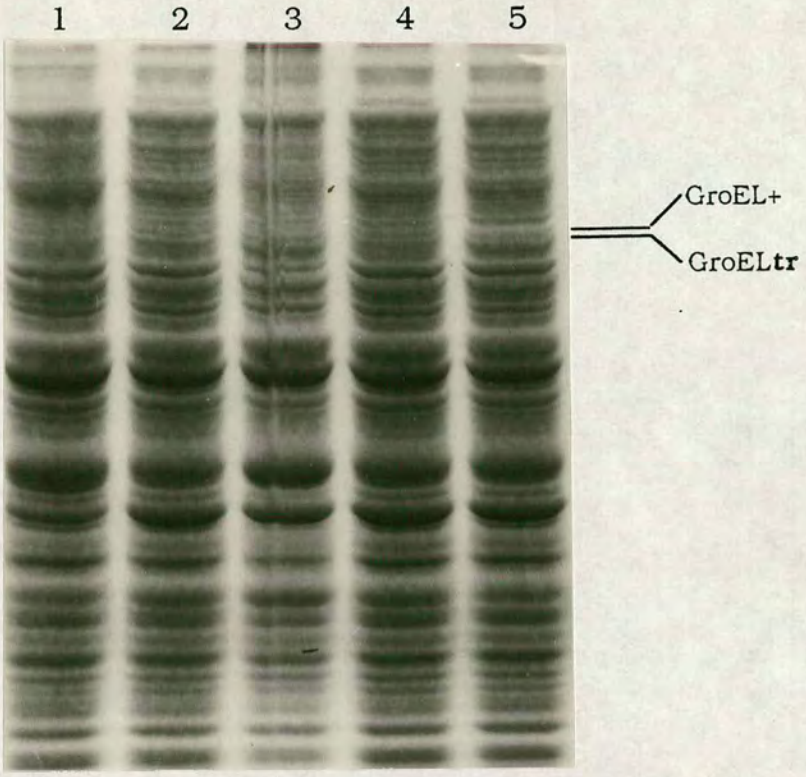
Strains were transduced with P1 ESLΔ::Ω selecting for *purA*$^+$ clones. Resulting transductants were patched onto plates +/– Str/Spc and incubated overnight at 30°C. Plates were then examined for growth and scored accordingly.

The strains generated in this experiment were used for further analysis. NM306 cells transduced to *purA*$^+$ were designated NL192. Those carrying the Ω fragment (and thus the *groE* deletion) were called NL192Ω.

4.5 Verification of the *groE* status of NL192 strains

In order to verify that the NL192 series of strains were indeed correct with respect to the nature of their *groE* genes their protein products were analysed by SDS–PAGE and their DNA examined using Southern blotting and DNA hybridization. Five strains were used in these analyses; NL192 carrying either pML31, pIRF1 or pHCF3 and NL192Ω carrying either pIRF1 or pHCF3. For SDS–PAGE analysis the strains were grown up overnight at 30°C and heat shocked for 30 minutes at 42°C, to encourage expression of the *groE* genes, prior to concentration by centrifugation and boiling in sample-cracking buffer. Samples were then subjected to SDS–PAGE. Examination of the gel showed that NL192(pHCF3) had two bands in the GroEL region, one corresponding to the wild-type protein and the other the truncated GroEL. NL192Ω(pHCF3) showed only one band in this region, corresponding to the smaller truncated protein and no trace of a wild-type GroEL protein was visible in this lane (Figure 4.5.1). Western blotting of these proteins and detection using affinity-purified anti-GroEL antibodies (kindly given by D. Young) also showed that no wild-type GroEL was present in samples that should be purely $GroEL_{tr}$, and in the lane containing both wild-type and truncated GroELs two bands can indeed be seen (Figure 4.5.2).

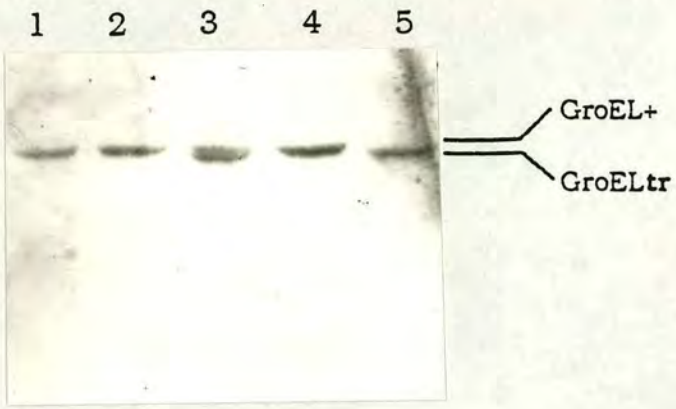
Figure 4.5.1 SDS–PAGE of NL192 strains.



1	NL192(pML31) whole-cell extract
2	NL192(pIRF1) whole-cell extract
3	NL192(pHCF3) whole-cell extract
4	NL192Ω(pIRF1) whole-cell extract
5	NL192Ω(pHCF3) whole-cell extract

Whole-cell extract were prepared as described in the *Materials and Methods*. Samples were electrophoresed using a 10% separating gel. The gel was stained with Coomassie brilliant blue. $GroEL^{+}$ and $GroEL_{tr}$ are marked. Close inspection of NL192Ω(pHCF3), lane 5, reveals an absence of full-length GroEL.

Figure 4.5.2 Western blot of NL192 strains using anti-GroEL antibodies.



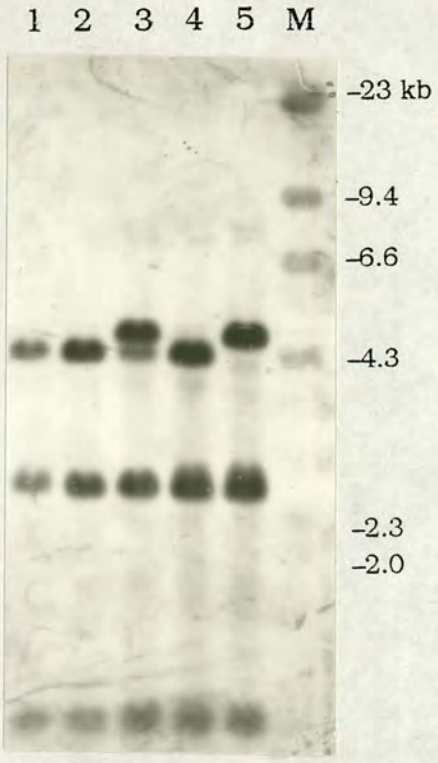
1	NL192(pML31) whole-cell extract
2	NL192(pIRF1) whole-cell extract
3	NL192(pHCF3) whole-cell extract
4	NL192Ω(pIRF1) whole-cell extract
5	NL192Ω(pHCF3) whole-cell extract

Samples and electrophoretic conditions were the same as that for Figure 4.5.1. The proteins were then electroblotted on to nitrocellulose, probed with anti-GroEL antibody and detected as described in the *Materials and Methods*. Note that NL192(pHCF3) produces two GroEL bands and NL192Ω(pHCF3) produces only truncated GroEL.

It is true that Western blotting/antibody detection of proteins is not an extremely sensitive technique, but since the amount of GroEL protein produced from a single-copy of the gene could be detected in all cases then the absence of a wild-type GroEL protein band with NL192Ω(pHCF3) suggests that this form of the protein is not produced.

For Southern blotting analysis DNA was prepared from these strains. Five micrograms of each was restricted using *Eco*RI/*Hin*dIII/*Pvu*II and electrophoresed through a 0.8% agarose gel. DNA in the gel was blotted onto a nitrocellulose membrane using the ammonium acetate transfer method of Smith and Summers (1980). The DNA on the filter was hybridized using biotin-labelled pND5, which carries the 8.1 kb *Eco*RI *groE* fragment, as a probe. It can be seen that the expected banding patterns were obtained in all cases (Figure 4.5.3).

Figure 4.5.3 Southern blot of NL192 strains' total DNA.



1	NL192(pML31) Total DNA
2	NL192(pIRF1) Total DNA
3	NL192(pHCF3) Total DNA
4	NL192Ω(pIRF1) Total DNA
5	NL192Ω(pHCF3) Total DNA
M	λ *Hin*dIII markers

Total DNA was restricted with *Eco*RI, *Hin*dIII and *Pvu*II, electrophoresed through a 0.8% agarose gel and blotted on to nitrocellulose as described in the *Material and Methods*. Biotin-labelled pND5 was used as the probe. A small amount biotin-labelled λ DNA was added to the hybridization mix in order to display the λ markers. See text below for details.

NL192(pML31) shows two major bands corresponding to ~2.7 and 4.3 kb with most of the *groE* operon located in the larger band. NL192(pIRF1) shows the same two bands but with slightly greater intensity owing to 2–3 *groE* copies per unit DNA compared to one with NL192(pML31). NL192(pHCF3) has the same two bands as before, but also has a band of ~4.6 kb owing to loss of the *Pvu*II site in pHCF3. The strains carrying *groE*Δ::Ω generate 2.7 and 2.8 kb fagments from

the chromosomal *groE* loci because of insertion of the Ω-fragment with its flanking *Hin*dIII sites. It can be seen that NL192Ω(pIRF1) also gives the 4.3 kb *groE* band, but NL192Ω(pHCF3) only gives the 4.6 kb *groES*$^{+}$ *EL*$_{tr}$ band.

Taken together these results show that the *groE* genes were deleted effectively during the construction of the NL192Ω strains, and that NL192Ω strain which carry pHCF3 and thus the GM-minus form as their sole GroEL species, do not have the capacity to produce a GM-containing version of the protein (which does not seem to cause the cells any problems under the conditions tested).

4.6 Summary and Discussion

Attempts have been made to examine the significance of the highly conserved GM tail of the GroEL protein. In *Chapter 3* it was shown that *groEL*$_{tr}$ could complement *groEL*^{ts} mutations. Since functional GroEL exists as a tetradecamer it seemed possible that the complementation observed was caused by the formation of heterogeneous tetradecamers containing both truncated and temperature-sensitive polypeptides. It was found that *groEL*$_{tr}$ could complement the *groE*-null mutation *groES3*::Ω. However, since this mutant contains an intact *groEL* sequence, which is potentially available for homologous recombination, it was decided that a true *groE* deletion strain should be constructed. The *groE* genes were replaced with the Ω fragment on a plasmid and this construction was used to replace the *groE* genes on the chromosome. Initial attempts, in which linear DNA was used to transform a *recD* strain, gave inexplicable results. All the potential positive clones turned out to have integrated the plasmid insert very close to, but not in, the *groE* operon. It is not obvious what recombinational event occurred in these cases but the phenomenon is not unique. It has been recently reported that there is a potential high-copy-number suppressor of *groEL*^{ts} mutations located just downstream of the chromosomal *Eco*RI restriction site distal to the *groE* operon (Greener *et al.*, 1993). It is possible that this gene contains some *groEL* sequence similarities.

A new method based on that of Kulakauskas *et al.* (1991) was used successfully to replace the chromosomal *groE* genes with the Ω fragment and the construction was verified by Southern blot analysis of the DNA. As with *groES3*::Ω, *groEL*$_{tr}$ could complement the new deletion *groE*Δ::Ω. Western blot analysis of the proteins produced by these cells showed that they contained only truncated GroEL. These cells were also found to grow at the same rate as isogenic cells carrying wild-type GroEL.

As stated previously, the conservation of the GM motif suggests that this tail would prove important for some aspect of GroEL function. It has now been shown unequivocally that no such function is required under the conditions we have tested. Nevertheless, since the products of the *groE* operon are probably required for many

different cellular functions, it is possible that the GM tail is required for some subtle aspects within the spectrum of GroEL's cellular repertoire. It was now decided that since strains carrying only $GroEL_{tr}$ are viable, a comparative study (*groEL*$^+$ versus *groEL*$_{tr}$) would be undertaken in order to find any phenotypic differences between strains carrying the two forms of the protein in the hope of obtaining an insight into some of GroEL's vital cellular functions.

Chapter 5

Attempts to elucidate the role of the GM tail in the GroEL protein—A search for a phenotype

5.1 Introduction

The generation of isogenic strains of *E. coli* that differed only in the form of GroEL (wild-type or truncated) they contained allowed investigation of the phenotypic properties associated with loss of the terminal GM motif. One would have thought that removal of such a highly conserved motif from an essential protein should lead to a reduction in its activity, however, since the GroE proteins are probably associated with several cellular functions, it may be that only a subset of those functions are affected. Indeed, removal of the GM tail did not appear to be detrimental under the conditions used in the construction of the mutant (glucose-supplemented minimal agar, 30°C); from this it can be inferred that the GM tail is dispensable for cellular viability and growth under these conditions.

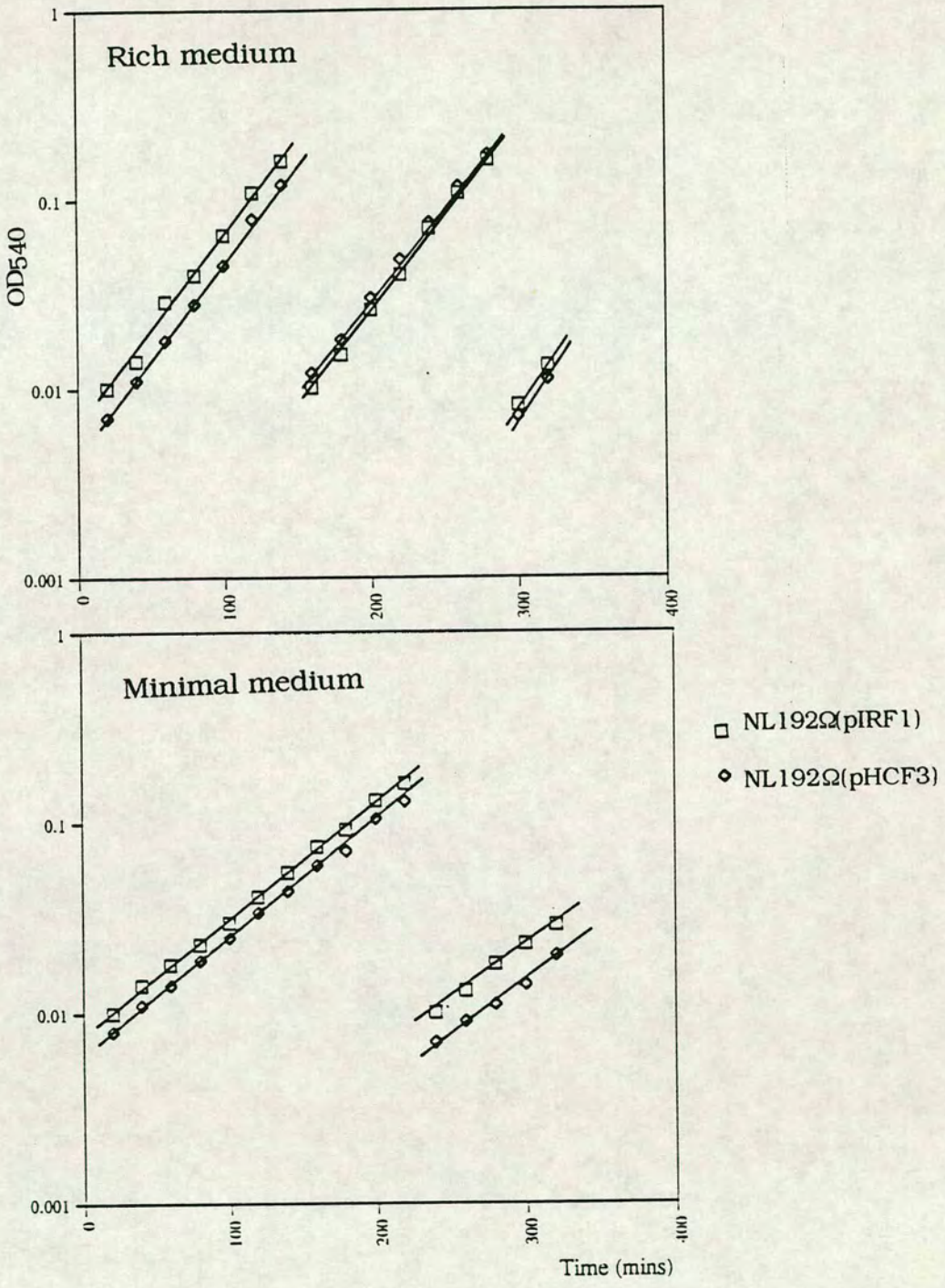
A variety of conditions were tested in which it was thought that the GroE proteins might play an significant role and these were used to compare the growth of the parent strain and the mutant expressing only *groEL*$_{tr}$. These included temperature, growth medium, bacteriophage sensitivity, and resistance to other stress-inducing agents.

5.2 Growth parameters of NL192Ω strains

The growth rates of the NL192Ω strains in different media at 37°C were investigated. Visual inspection of colonies of NL192Ω carrying either pIRF1 or pHCF3 growing at 37°C on L-agar plates or minimal agar plates showed that the strains produced colonies of similar sizes. However, since closer analysis of the growth rates of these strains may have revealed differences between them, growth rates of liquid cultures were measured. NL192Ω(pIRF1) and NL192Ω(pHCF3) were diluted 1:100 from overnight cultures into fresh L-broth or VB-supplemented minimal liquid medium. The strains were grown at 37°C and the optical densities of the cultures followed at 540 nm. The OD_{540} of the cultures were maintained between 0.01 and 0.2 by dilution into prewarmed medium (to maintain constant growth rates). As can be seen, no differences in growth rates were found between the strains in either the rich or minimal media indicating that NL192Ω

carrying GroEL$_{tr}$ is not compromised in its growth rate potential at 37°C compared to GroEL$^+$ (Figure 5.2.1).

Figure 5.2.1 Growth curves of NL192Ω pIRF1/pHCF3 at 37°C.



For experimental details see text.
Doubling times,rich medium
NL192Ω(pIRF1), 32 ± 3 min, NL192Ω(pHCF3), 33 ± 3 min
Doubling times,min medium,
NL192Ω(pIRF1), 50 ± 4 min, NL192Ω(pHCF3), 51 ± 4 min

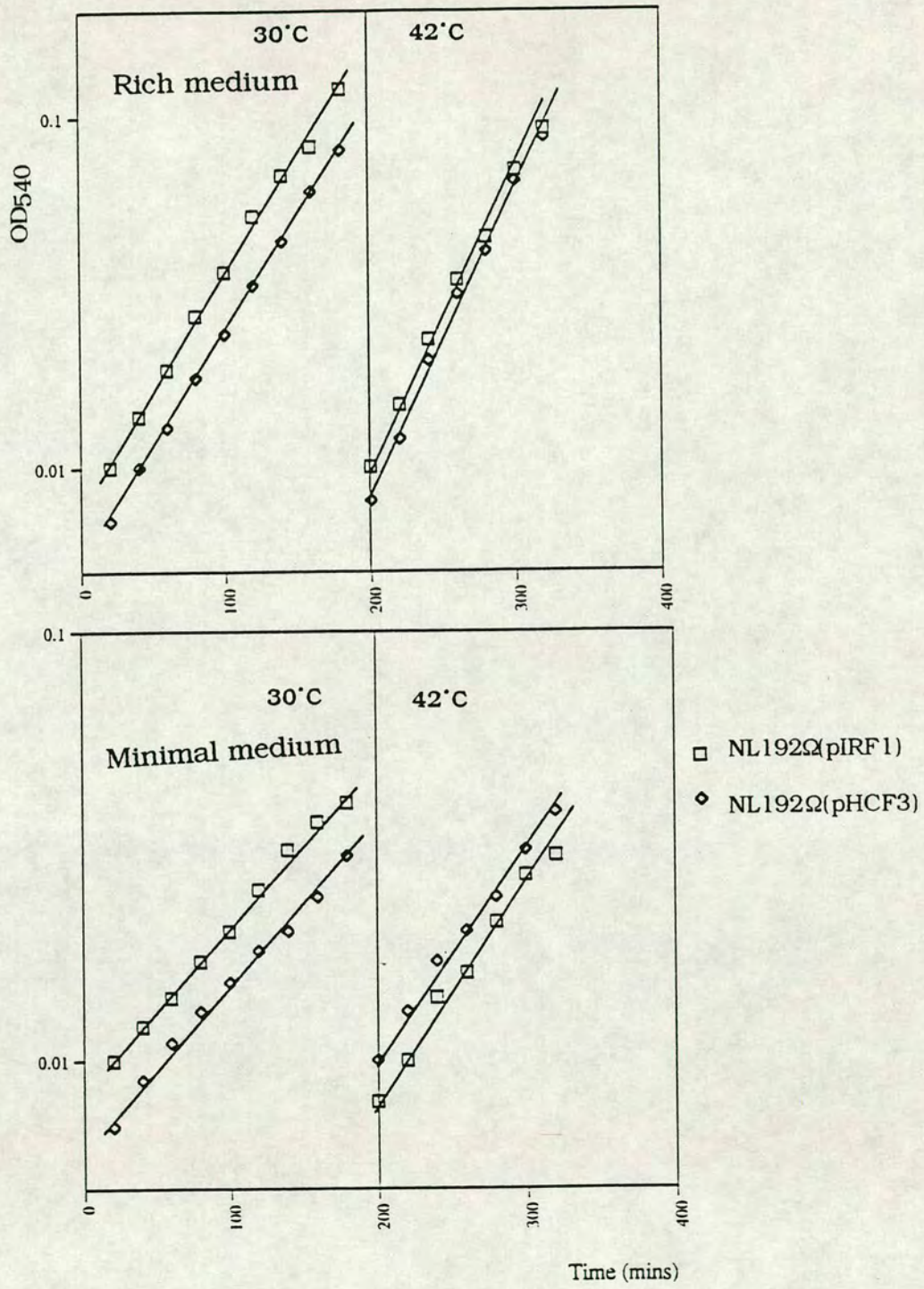
5.3 Effects of environmental factors

5.3.1 Effects of temperature

The GroE proteins are essential for viability at all growth temperatures but are further induced by heat shock. At its peak GroEL can represent greater than 10% of total cell protein and it is almost certainly important at high temperature. Indeed, the GroE proteins are the only HSPs that need to be overexpressed in order to raise the maximum growth temperature of an *rpoH* deletion strain (coding for σ-32, the HSP transcription factor) from 20°C to 40°C, again showing their importance for survival at physiological growth temperatures (see *Chapter 1*). Temperature-sensitive *groE* strains have been reported to show a variety of growth defects at their non-permissive temperatures, including impaired RNA and DNA synthesis (Wada and Itikawa, 1984), and an inhibition of cell division leading to the formation of filaments (Georgopolous and Eisen, 1974).

To test the effects of temperature on cellular growth and viability, NL192Ω pIRF1(GroEL$^+$) and NL192Ω pHCF3(GroEL$_{tr}$) were streaked out on agar containing rich (L-broth) or minimal medium (VB minimal plus glucose) at 30, 37 and 42°C, and incubated overnight. Examination of the plates revealed no differences between the strains with regard to the size and numbers of colonies formed. As a more accurate measure growth curves of liquid cultures involving a temperature shift from 30 to 42°C were made for the two strains. The strains were grown at 30°C and the optical densities of the cultures followed at 540 nm. The OD_{540} of the cultures were maintained between 0.01 and 0.2 by dilution into medium prewarmed at 30°C (to maintain constant growth rates). At a given time the strains were diluted into medium prewarmed at 42°C and the OD_{540} of the cultures were followed at this temperature. Again no significant differences could be seen between the two strains (Figure 5.3.1).

Figure 5.3.1 Growth curves of NL192Ω pIRF1/pHCF3 at 30°C with 30–42°C temperature shift.



For experimental details see text.
Doubling times,rich medium, NL192Ω(pIRF1), 30°C = 44 ± 3 min, 42°C = 34 ± 3 min.
NL192Ω(pHCF3), 30°C = 45 ± 3 min, 42°C = 34 ± 3 min.
Doubling times,min medium, NL192Ω(pIRF1), 30°C = 82 ± 7 min, 42°C = 63 ± 4 min.
NL192Ω(pHCF3), 30°C = 83 ± 7 min, 42°C = 67 ± 4 min.

A high temperature survival test was also performed. *E. coli* is rapidly killed when shifted from 30 to 56°C, however, survival improves when the culture is allowed to remain at 42°C for some time prior to the 56°C challenge (Yamamori and Yura, 1982). This is a transient effect which is at a maximum after 30 minutes of preincubation, and disappears after one hour. Although it has now been shown that σ-E regulates the genes primarily responsible for thermotolerance (Erickson and Gross, 1989) σ–32 depleted cells also show a lack of thermotolerance (Jenkins *et al.*, 1991). An attempt was made to see whether cells carrying the truncated GroEL were more sensitive to high-temperature treatment than those carrying the wild-type protein.

NL192(pML31), NL192Ω(pIRF1) and NL192Ω(pHCF3) were grown up to mid-log phase at 30°C. The cells were then diluted to give approximately 10^3 cells ml^{-1}. Aliquots of each strain were incubated for 30 minutes at 30 and 42°C. The cells were then transferred to 56°C and aliquots removed at various times and plated onto L-agar and incubated at 30°C overnight. Again no major differences were seen between the two NL192Ω strains in either survival rate at 56°C or the protection offered by preincubating at 42°C (Table 5.3.1).

Table 5.3.1 High temperature survival.

	Plating efficiency after incubation at 56°C for (min)				
Strain	**0**	**5**	**10**	**15**	**30**
NL192(pML31)	1.0	$2x10^{-1}$	$3x10^{-2}$	$5x10^{-3}$	$6x10^{-4}$
NL192Ω(pIRF1)	1.0	$6x10^{-2}$	$3x10^{-3}$	$1x10^{-3}$	$1x10^{-4}$
NL192Ω(pHCF3)	1.0	$4x10^{-2}$	$2x10^{-3}$	$1.5x10^{-3}$	$1x10^{-4}$

Strains were grown in L-broth to mid-log phase at 30°C before transfer to 56°C. Aliquots were removed at the indicated times and serial dilutions of these were plated out at 30°C. Values given refer to the fraction of viable colony-forming cells relative to the initial number of cells treated.

High temperature survival after preincubation at 42°C.

	Plating efficiency after incubation at 56°C for (min)				
Strain	**0**	**5**	**10**	**15**	**30**
NL192(pML31)	1.0	$6.5x10^{-1}$	$9.5x10^{-2}$	$1x10^{-2}$	$1.5x10^{-3}$
NL192Ω(pIRF1)	1.0	$2x10^{-1}$	$1x10^{-2}$	$2x10^{-3}$	$2x10^{-4}$
NL192Ω(pHCF3)	1.0	$2x10^{-1}$	$1.5x10^{-2}$	$2x10^{-3}$	$2x10^{-4}$
(preincubated at 42°C)					

Experimental detail as above except the cells were preincubated at 42°C for 30 minutes prior to 56°C transfer.

It was noted that NL192(pML31), carrying *groE* on the bacterial chromosome, fared better than the NL192Ω strains, which carry *groE* on mini-Fs. The reason for this is unclear but one possibility is that the Str^R/Spc^R of cells carrying the Ω fragment are impaired in some way after exposure to 56°C, leading to a proportion of the cells dying when plated on antibiotic containing agar. However, since the NL192Ω strains producing either GroEL$^+$ or GroEL$_{tr}$ behaved similarly this was not investigated further .

Taken together these results indicate that cells carrying the truncated form of GroEL performed no worse than did their isogenic wild-type counterparts when faced with changes in temperature. Nevertheless, since temperature is thought to be one of the factors

regulating levels of GroE proteins within the cell, a range of temperatures continued to be used in subsequent comparative tests.

5.3.2 Effects seen with a variety of carbon sources

Strains carrying either the truncated or wild-type GroEL proteins were tested for their ability to utilize a range of carbon sources. Earlier experiments had employed either a rich medium packed-full of amino acids and vitamins, or a minimal medium with glucose as the carbon source. Work by Van Dyke *et al.* (1989) has suggested a role for the GroE proteins in histidine (and probably other amino acid) biosynthesis. Since the truncated GroEL-bearing strain grew as well as the control on minimal medium, it can be concluded that the amino acid biosynthesis proteins (and all other essential biosynthesis proteins involved in metabolism and cellular maintenance) in the truncated strain are sufficiently active to supply cellular needs and thus amino acid assimilation does not absolutely require a GM-bearing GroEL. Unfortunately the strains used here are histidine minus (*hisG*) and the need for GroEL containing the GM tail in histidine biosynthesis was not tested.

We then tested whether carbon source metabolism was adversely affected. The separate provision of diverse carbon sources would require the activation of various multi-enzyme operons normally dormant during growth on glucose. NL192Ω(pIRF1) and NL192Ω(pHCF3) were streaked out on minimal agar containing a variety of carbon sources. These were galactose, maltose, mannose, melibiose, ribose, raffinose, glycerol, mannitol, sorbitol, succinate, proline/alanine and glucose as a control and were present in the plates at a concentration of 0.2%. Plates were incubated at 30 and 42°C overnight or longer if required. As can be seen in Table 5.3.2 no differences were seen between the growth of strains on the carbon sources tested.

Table 5.3.2 Effects of alternative carbon sources.

	Strain			
	NL192Ω(pIRF1)		NL192Ω(pHCF3)	
Carbon Source	**30°C**	**42°C**	**30°C**	**42°C**
Glucose	+++	+++	+++	+++
Galactose	+++	+++	+++	+++
Maltose	+++	+++	+++	+++
Melibiose	++	+/-[a]	++	+/-[a]
Ribose	+++	+++	+++	+++
Raffinose	++	+	++	+
Glycerol	+++	++	+++	++
Mannitol	+++	+++	+++	+++
Sorbitol	+++	++[b]	+++	++[b]
Succinate	+++	+++	+++	+++
Proline/Alanine	+	-	+	-

Strains were streaked onto VB minimal agar (with the required amino acid supplements) plus the given carbon source and incubated at 30 and 42°C until colonies had formed (usually up to 48 h). +++, well formed colonies/-, no colonies visible.

a. Very poor growth. The stains are *lacY*. LacY is required for melibiose transport at 42°C.

b. Reduced numbers of colonies compared to 30°C.

5.4 Bacteriophage sensitivity of NL192Ω strains

GroE mutants of *E. coli* were originally isolated by UV mutagenesis followed by selection for bacteriophage T4- or λ-resistant clones. These *groE* mutants were shown to be blocked at the level of phage particle assembly. They were also shown to be temperature sensitive at 42°C as well as phage resistant at permissive temperatures; *groEL* mutants are resistant to phages T5 and φ80 in addition to T4 and λ. The ability of the truncated *groEL* strain to support growth of T4, T5 and λ (unfortunately phage φ80 could not be obtained) was therefore tested. NL192Ω pIRF1 and pHCF3 were used to make bacterial lawns in BBL top-agar on L-agar plates. Various dilutions of the phages were spotted onto the top agar lawns and the plates incubated overnight at 30 and 42°C. Examination of the plates showed no differences between the strains; the numbers and size of plaques obtained were identical. These results highlight two points. First, bacteriophages that require host GroEL function for their propagation can effectively utilize the GM-lacking form of the protein, and second, that the mutations in *groEL* which abolish bacteriophage propagation are probably not located in the GM motif-encoding region of the gene. Again the GM tail is dispensible for function.

5.5 Effects of stress on NL192Ω strains

Despite the fact that temperature seemed to have little or no effect on the strains expressing *groEL*$_{tr}$, it was decided to test whether strains producing either GroEL$^+$ or GroEL$_{tr}$ would respond differently to other stress-inducing agents.

5.5.1 Ethanol stress

Ethanol is a potent inducer of the heat-shock response and most (possibly all) HSPs that are induced by heat are also induced by exposure to ethanol (see Neidhardt and VanBogelen, 1987; Travers and Mace, 1982). In order to determine whether GroEL's GM tail was important for GroE function during ethanol stress, NL192Ω carrying either pIRF1 or pHCF3 was streaked out on L-agar plates containing between 0–8% ethanol and incubated at 30 and 42°C for up to 3 days (growth on ethanol-containing plates is very slow). Growth was then compared. As the results in Table 5.5.1 show, both strains plated equally well (or badly) on the given medium.

Table 5.5.1 Ethanol plating NL192Ωs.

		Ethanol Concentration					
		0%		4%		8%	
Strain	**Plasmid**	30°C	42°C	30°C	42°C	30°C	42°C
NL192Ω	pIRF1	+++	+++	+++	++	-	-
	pHCF3	+++	+++	+++	++	-	-

Strains with the given plasmids were streaked out on L-agar with various concentrations of ethanol at the stated temperatures. +++, well formed colonies; -, no colonies visible.

In order to determine whether *groE* gene dosage affected the ability of strains to grow on ethanol-containing medium, the NL192Ω strains were transformed with *groEL*$^+$ and *groEL*$_{tr}$ encoding multi-copy plasmids. NL192Ω(pIRF1) was transformed with pGTIR88 to give overexpression of *groEL*$^+$, and NL192Ω(pHCF3) with pGTHC18 to overexpress *groEL*$_{tr}$. These strains were streaked out as before on

ethanol-containing L-agar plates and incubated at 30 and 42°C for a suitable period of time. The results seen in Table 5.5.2 show that once again there were no apparent differences between the strains, however, at 42°C with 4% ethanol neither grew as well as the parent strains.

Table 5.5.2 Ethanol plating NL192Ωs overexpressing *groEL*$^+$/*groEL*$_{tr}$.

		Ethanol Concentration					
		0%		4%		8%	
Strain	**Plasmids**	30°C	42°C	30°C	42°C	30°C	42°C
NL192Ω	pIRF1+						
	pGTIR88	+++	+++	+++	+	-	-
	pHCF3+						
	pGTHC18	+++	+++	+++	+	-	-

Strains with the given plasmids were streaked out on L-agar with various concentrations of ethanol at the stated temperatures. +++, well formed colonies; -, no colonies visible.

Under these rather stressful conditions, overexpression of the *groE* genes causes cells to grow more slowly, perhaps because of interference with the normal stress-response damage-limitation pathway. However, this response does not appear to be affected by the absence of the GM tail.

5.5.2 Resistance to UV irradiation

Mutations within *groE* have been shown to reduce the frequency of UV-induced mutagenesis in *E. coli* (Donnelly and Walker, 1989). It has also been shown that the GroE proteins (and other HSPs) are induced after UV irradiation in a *rpoH*-dependent manner (Kruger and Walker, 1984). It can therefore be assumed that all σ-32 regulated HSPs will be induced when the cell is responding to DNA damage. It has been suggested that DNA damage itself, with the concomitant relaxation of DNA supercoiling, could be the primary inducer of the heat-shock proteins (Travers and Mace, 1982). In a simple UV-resistance test, NL192Ω(pIRF1) and NL192Ω(pHCF3) were streaked across L-agar

plates and subjected to increasing doses of UV across the streak. NL44 (*groEL44*) and NL30 (*groES30*) were also included in the test as negative controls. The plates were incubated overnight at 30, 37 and 42°C and examined for growth. As shown in Table 5.5.3, both the NL192Ω strains grew equally well at all the temperatures tested, whereas NL30 and NL44 fared worse at both 30 and 37°C (NL30 and NL44 do not grow at 42°C).

Table 5.5.3 UV resistance of NL strains.

Strain	30°C	37°C	42°C
NL30	45	30	na
NL44	45	30	na
NL192Ω pIRF1	60	60	70
NL192Ω pHCF3	60	60	70

Strains were irradiated with UV light as indicated in the *Materials and Methods* and the plates incubated at the given temperature. The numbers reflect the exposure time in seconds before a significant loss (as judged by myself) of viability was observed.
na. Not applicable.

This confirms that the GroE proteins are indeed required for strains to withstand the full effects of UV irradiation; *groE*^{ts} mutants become irreparably damaged by shorter exposure to UV irradiation than strains producing wild-type GroE proteins. The observation that the strain carrying *groEL*$_{tr}$ survived UV as well as the wild-type strain shows that GroEL$_{tr}$ is not defective in UV-recovery functions. It was also noticed that when the strains were incubated for recovery at 42°C they could withstand slightly higher doses of UV irradiation compared to those incubated at 30 and 37°C. Perhaps the greater abundance of certain HSPs at the higher temperature can improve the cells' recovery.

5.5.3 Resistance to streptomycin

March (1988) did an extensive study into the effects of overproduction of the GroE proteins on antibiotic resistances. The most striking effect

seen was a heightened resistance to the antibiotic streptomycin when the *groE* genes were present in high copy number. Streptomycin can induce a heat-shock response (see Neidhardt and VanBogelen, 1987), and the effect was even more pronounced at high temperatures. Importantly it was shown that overproduction of the GroE proteins does not in itself confer streptomycin resistance, but rather increases the levels of resistance in strains already resistant to the drug (by virtue of the *rpsL* mutation producing a ribosomal protein that has a reduced affinity for streptomycin). Control strains were resistant to no more than 800 μg ml^{-1} of streptomycin both at 30 and 42°C. When the GroE proteins were overproduced from pND5 growth could be seen at concentrations above 1200 μg ml^{-1} at both 30 and 42°C, with the growth at 42°C being considerably better than at 30°C. Streptomycin is known to affect translation initiation and elongation by binding to the 30S ribosomal subunit, which leads to aberrant polypeptide production. It is possible that in the presence of large amounts of the antibiotic the GroE proteins physically block streptomycin binding to the RpsL protein, especially under heat-shock conditions (i.e. 42°C). GroEL is known to copurify with ribosomes (both in *E. coli* and S. *cerevisiae*) and the possibility of an interaction between GroE proteins and ribosomes has never been ruled out (Subramanian *et al.*, 1976; Neidhardt *et al.*, 1981; R. Hallberg, personal communication). Perhaps the GroE proteins prevent thermal denaturation of ribosomes during heat shock. Another possibility for explaining this effect is that strains which overproduce the GroE proteins are better equipped to cope with the large amounts of aberrant polypeptides produced when high concentrations of streptomycin are present.

It was decided to determine what effects streptomycin has upon strains carrying the truncated form of GroEL. NL192Ω (which possesses the Str^R *rspL* mutation) carrying pIRF1 (*groE*$^+$) was transformed with the high-copy-number plasmids pGTIR88 (*groE*$^+$) or pJF118 (as a control). NL192Ω(pHCF3) (*groES*$^+$, *EL*$_{tr}$) was transformed with pGTHC18 (*groES*$^+$, *EL*$_{tr}$) or pJF118 (as a control). These strains were streaked out onto L-agar plates containing various levels of streptomycin (0–1500 μg ml^{-1}) at 30 and 42°C. Unexpectedly it was found that all strains were resistant to the highest concentration of streptomycin at both temperatures. This was in disagreement with

March's results (1988) so I decided to use even higher concentrations of the drug in the expectation that an effect would be seen. All the above strains were plated out on medium containing up to 5000 μg ml^{-1} streptomycin at 30 and 42°C. As before all the strains grew at both temperatures apparently equally well, as judged by visual inspection of the plates. It is unclear why these strains were growing on streptomycin concentrations that are more than 8 times greater than those observed by March. I decided to repeat the work of March using the same plasmids and Str^R strain MM18. MM18 was transformed with pBR325, pND5, pGTIR88 and pGTHC18. Each strain was plated on L-agar containing 0–3000 μg ml^{-1} streptomycin and incubated at 30 and 42°C. This time I did indeed see an effect, however, it was the complete opposite to that obtained by March! I found that cells carrying pBR325, pGTIR88 and pGTHC18 grew well on all concentrations of streptomycin tested whereas cells containing pND5 grew poorly on 750–1000 μg ml^{-1} streptomycin and not at all on 1250 μg ml^{-1} at 42°C. The growth of MM18(pND5) at 30°C was comparable to that of the other plasmid-containing strains (Table 5.5.4)

Table 5.5.4 Streptomycin resistance of MM18.

Streptomycin	**MM 18**							
conc. (μgml^{-1})	**pBR325**		**pND5**		**pGTIR88**		**pGTHC18**	
	30°C	42	30	42	30	42	30	42
250	+++	+++	+++	+++	+++	+++	+++	+++
750	+++	+++	+++	++[a]	+++	+++	+++	+++
1000	+++	+++	+++	+[a]	+++	+++	+++	+++
1250	+++	+++	+++	-	+++	+++	+++	+++
1500	+++	+++	+++	-	+++	+++	+++	+++
3000	++	+++	++	-	++	+++	++	+++

MM18 was transformed with the given plasmids and streaked out on L-agar containing 0–3000 μg ml^{-1} streptomycin and incubated at both 30 and 42°C. Plates were then examined for growth. +++, well formed colonies. -, no colonies visible.
a. Small colonies and greatly reduced in numbers.

It is hard to comment upon such a result but at least it can be stated that cells carrying pGTIR88 (*groEL*$^+$) and pGTHC18 (*groEL*$_{tr}$) behave identically when exposed to high levels of streptomycin. This was not the only inexplicable result obtained with pND5 (see below).

5.5.4 Resistance to nalidixic acid

In the course of his investigations on the effects of *groE* overexpression, Jenkins discovered that strains resistant to the antibiotic nalidixic acid (Nal^R) were made nalidixic acid sensitive (Nal^S) in the presence of plasmid pND5 (Jenkins, 1985). The strain used in this experiment was MM7 which is Nal^R by virtue of the presence of a *gyrA* mutation. *GyrA* encodes one subunit of the DNA gyrase protein, the target for nalidixic acid, an interaction which blocks subsequent DNA replication. This work was expanded by March (1988) who showed that MM7 (pND5) grew more poorly with increasing amounts of the drug, but recovered when transferred to drug-free medium. It was also shown that plasmid-free MM7 shows a growth lag when challenged with nalidixic acid, and that this lag is increased by temperature and by the presence of pND5. Nalidixic acid exposure induces the GroE proteins (along with other HSPs) in a σ32 dependent fashion (Kruger and Walker, 1984). This, plus the fact that the pND5-induced nalidixic acid sensitivity was more apparent at high temperatures led March to the conclusion that it was indeed the GroE proteins produced from pND5 that were causing this effect.

In view of this it seemed reasonable to find out if an effect on nalidixic acid resistance could also be demonstrated with GroEL$_{tr}$. MM7(*gyrA*) was transformed with pGTIR88 (*groE*$^+$), pGTHC18 (*groES*$^+$, *EL*$_{tr}$) and pJF118 (as a control). Transformants were then streaked out on L-agar plates with various amounts of nalidixic acid (0–40 μg ml^{-1}) at 30 and 42°C. The original experiments performed by Jenkins employed 20 μg ml^{-1}. On examination of the incubated plates no differences between any of the plasmid-bearing strains were seen. This rather surprising result led to repeating the experiment with higher concentrations of nalidixic acid. This time the plates contained up to 200 μg ml^{-1} nalidixic acid. Again no differences were observed in that all the strains grew more or less equally well on all of the plates at both of the temperatures. At this stage I was worried about the

validity of both Jenkin's and March's observations, but decided to repeat the experiment with the original plasmid pND5. MM7 was transformed with pND5 and streaked out on L-agar plates containing 0–100 μg ml^{-1} nalidixic acid again at 30 and 42°C. The other MM7 transformants were also streaked out. As before MM7 carrying pJF118, pGTIR88 and pGTHC18 grew on all concentrations of nalidixic acid, but MM7 with pND5 did not form colonies on plates with a nalidixic acid concentration greater than 20 μg ml^{-1} at 42°C. Growth at 30°C was comparable to the other strains. This showed that the original observations were correct, but the conclusions gleaned from those observations were probably wrong. Plasmid pND5 carries the *groE* genes near the middle of an 8.1 kb *Eco*RI chromosomal DNA fragment. The *groE* operon is encoded by only 2.1 kb and so there is much scope for the production of other polypeptides from this DNA insert. Mini-cell analysis has shown that pND5 does indeed produce proteins other than the GroE proteins and those produced by its parent plasmid pBR325. These include proteins with a molecular mass of 38, 32, 18 and 13.5 kDa (Jenkins *et al.*, 1986). As far as is known none of these proteins has been identified, sequenced, accurately mapped or characterized. The *groE* containing plasmids (pGTIR88 and pGTHC18) that I used in this experiment carry a 5 kb chromosomal DNA insert, and thus there is about 3 kb of DNA present in pND5 that is not present in pGTIR88. Could it be that there is a gene within this region that is producing the nalidixic acid-sensitivity effect rather than *groE*? The above data certainly suggests that nalidixic acid resistance in *gyrA* strains is unaffected by the overproduction of the GroE proteins, be they wild-type or truncated. The possibility that this effect was caused by some other protein encoded by the 8.1 kb chromosomal insert could be tested. Plasmid pIR88 carries the same 8.1 kb *groE*$^+$ DNA fragment as pND5, and a derivative of pIR88, pHCΩ1 also carries this insert but is deleted for *groE*. If both these plasmids have the same debilitating effect as pND5 on nalidixic acid resistance then the involvement of the *groE* genes can be ruled out. MM7 was transformed with pIR88 (*groE*$^+$), pHC18 (*groES*$^+$, *EL*$_{tr}$), pHCΩ1 (*groE*::Ω) and pVH1 (as a control). The transformed cells were then streaked out on L-agar containing 0–100 μg ml^{-1} nalidixic acid (as well as antibiotics for plasmid selection) at

30, 37 and 42°C as were all the other MM7 plasmid-carrying strains mentioned previously. It was found that MM7 carrying pVH1, pIR88, pHC18, pHCΩ1, pJF118, pGTIR88 and pGTHC18 were equally resistant to 100 μg ml^{-1} nalidixic acid at all the temperatures tested; only the strain carrying pND5 failed to grow.

Previous work showed that pND5's parent plasmid pBR325 does not cause nalidixic acid sensitivity suggesting that the effect is caused by the presence of the 8.1 kb *groE*-containing insert. However pIR88, which contains the same 8.1 kb DNA fragment actually derived from pND5, does not cause nalidixic acid sensitivity. None of the other plasmids tested, which contain various parts of the 8.1kb insert, causes the effect either. SDS–PAGE analysis of strains carrying each of these *groE*-containing plasmids also show that they all overproduce GroEL at levels similar to that seen with pND5, suggesting that GroEL concentration is not important. It seems that nalidixic acid sensitivity seen occurs with pND5, although the involvement of the *groE* genes in this reaction, as suggested by Jenkins (1985) and March (1988), can now be excluded. Since it was found that the GroE proteins had no effect upon nalidixic acid resistance in MM7, the sensitivity caused by pND5 was not studied further.

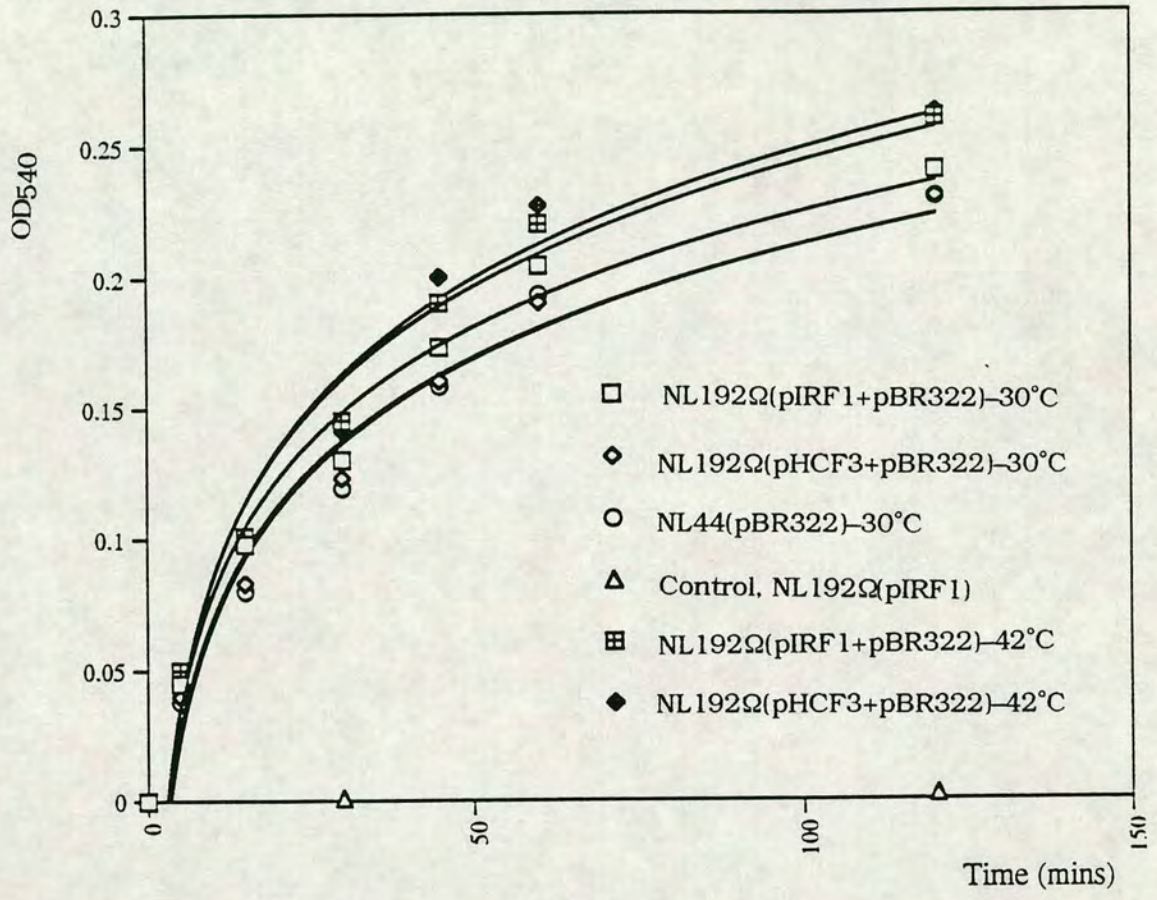
5.6 Effects on β-lactamase excretion

Bochkareva *et al.* (1988) showed that newly synthesized β-lactamase is bound *in vitro* by GroEL, and that the bound peptides are subsequently translocated into inverted membrane vesicles on addition of ATP. This work was followed up with reports that β-lactamase excretion into the periplasm *in vivo* is retarded in *groE* mutants, more so in *groES* than in *groEL* mutants (Laminet *et al.*, 1990). It was therefore decided to attempt to quantify the amount of β-lactamase excreted in truncated GroEL-bearing and wild-type cells. NL192Ω pIRF1 and pHCF3 were transformed with pBR322 using tetracycline as the selective agent. These cells were grown up at both 30 and 42°C to an OD_{540} of 0.5, and, after washing, subjected to osmotic shock to prepare a periplasmic extract (Neu and Heppel, 1965); a whole-cell extract was obtained by sonication. As a control a culture of strain W3110 was induced with 5 μg ml^{-1} IPTG and its extracts assayed for β-galactosidase, a protein which should not be found in the periplasm. The induced W3110 extracts showed that there was four times more β-gal activity in the whole-cell extract than in the periplasmic fraction and thus that the technique worked reasonably well. Uninduced W3110 showed about ten times more β-gal activity in the whole-cell extract compared to the periplamic fraction (data not shown). The β-gal activity seen in the periplasmic fraction was probably caused by cytoplasmic contamination from lysed cells.

The assay for β-lactamase involves use of 87/312 (nitrocefin), which is a chromogenic cephalosporin that produces a red colour when hydrolysed by β-lactamase. In all cases the levels of β-lactamase were as high in the periplasmic fraction as they were in the whole-cell extract, indicating either that β-lactamase is translocated very efficiently, or that pre-β-lactamase, which has not yet been translocated, is inactive. It could be argued that the cells grown at 42°C had greater levels of β-lactamase in their periplasmic fractions compared to the samples grown at 30°C, but comparisons between strains carrying GroEL$_{tr}$ and GroEL$^+$ showed no marked differences with comparable rate kinetics and maximum values (Figure 5.6.1). In retrospect this is not surprising since it has been shown that *groEL*

mutants are only marginally affected in their ability to translocate β-lactamase (Laminet *et al.*, 1990).

Figure 5.6.1 Hydrolysis of nitrocefin by β-lactamase-containing periplasmic extracts from NL strains.



Osmotic-shock cold-water washes isolated from cells grown at 30 and 42°C (see the *Materials and Methods*) were diluted 100x in 0.01 M phosphate buffer and nitrocefin was added at a final concentration of 40 μg ml^{-1}in this case. The final reaction volume was 1 ml and the OD_{540} of the reaction was measured and plotted as a function of time. Different concentrations of nitrocefin and cold-water washes always gave similar results when comparing the different strain used, and this is only one example of such an experiment. The samples were comparable because the OD_{540} of the original cultures were identical. It should be noted that the reaction was kept dark as this appeared to give better colour development. (Based on the method of O'Callaghan *et al.*, 1972.)

5.7 Isolation of total membranes from GroEL$^+$ and GroEL$_{tr}$ overproducing cells

One of our original hypotheses on the potential role of the GM motif in GroEL was that this apparently hydrophobic sequence may be acting as a membrane anchor. If this were the case, less GroEL$_{tr}$ than GroEL$^+$ would be found in membrane preparations. To examine this I isolated total membranes from cells overproducing either GroEL$^+$ or GroEL$_{tr}$. NL192Ω(pIRF1+pGTIR88) and NL192Ω(pHCF3+pGTHC18) were grown at 30 and 42°C for the preparations. The isolated membrane fractions were then analysed by SDS–PAGE. Figure 5.7.1 shows the membrane fractions isolated from cells grown at 42°C.

It can be seen that these fractions contain little GroEL. Considering that these cells were overproducing the GroE proteins and the fact that very little of the protein is seen in these membrane preparations suggests that either GroEL is not at all membrane associated, or there is, at best, a weak association which cannot withstand the rigours of membrane isolation. It can be seen, however, that the levels of GroEL$^+$ and GroEL$_{tr}$ are similarly low. In fact it is only the size difference between GroEL$^+$ and GroEL$_{tr}$ that identifies the GroEL proteins on the gel.

One interesting observation from this gel was that in both strains GroEL seems to exist as two distinct bands with the smaller one being more adundant in both cases. I cannot say which of these represents the 'normal' form of GroEL. Spangfort *et al.* (1993) has also reported finding two species of GroEL in highly purified preparations and suggests that the larger form might be phosphorylated, although evidence is now accumulating against this idea (M. Spangfort, personal communication). I do not know the nature of the second form of GroEL, but from my gels it can be said that if the smaller band is a processed form of the protein then the processing must have occurred at the amino-terminus since GroEL$_{tr}$ shows a similar displacement to GroEL$^+$ on the gel; alternatively it may be caused by internal modification. As far as I am aware, there have been no reports of processed forms of the GroEL proteins being detected in *E. coli*, although there are reports of some cells which have multiple *groEL* genes and proteins (Guglielmi *et al.*, 1991; Mazodier *et al.*,

1991; Rinke de Wit *et al.*, 1992; P. Lund personal communication). It is not inconceivable that a processed form of GroEL is required in *E. coli* at levels that are not normally detected and indeed a phosphorylation of GroEL has been shown to affect substrate binding and release *in vitro* (Sherman and Goldberg, 1992). Phosphorylation could account for the size difference seen between the two GroEL bands in my preparations, approximately 3–5 kDa, which is similar to that described by Sherman and Goldberg (1992). However, further investigation would be required to comment informatively on this observation.

Figure 5.7.1 SDS–PAGE of membrane proteins isolated from NL192Ω overproducing GroEL^{+}/GroEL$_{tr}$ at 42°C.

1	NL192Ω (pIRF1+pGTIR88)
2	NL192Ω (pHCF3+pGTHC18)
M	Molecular mass markers

Total membranes were isolated from NL192Ω (pIRF1+pGTIR88) and NL192Ω (pHCF3+pGTHC18) grown at 42°C as described in the *Materials and Methods*. Note the two bands which are displaced in lane 2 relative to lane 1 (➔).

5.8 Effects seen in combination with *dnaA46* mutation

The only difference found so far in the search for a role of the GM tail of GroEL is that the truncated version of the protein cannot suppress temperature-sensitive *dnaA* mutations that can be suppressed by overexpression of the wild-type protein. This lack of high temperature growth is associated with the lack of the cold sensitivity found in the suppressed strains. The suppression is thought to be caused by an interaction between the GroE proteins and the mutant DnaA, which might indicate that GroEL and DnaA normally interact *in vivo*. If truncated GroEL and the mutant DnaA are failing to interact, a strain that carries both *dnaA46* and *groEL*$_{tr}$ may be disabled. I therefore constructed a *dnaA46* derivative of NL192Ω.

In order to transfer the *dnaA46* gene into the NL192Ω strains a transposon, Tn*7* (encoding trimethoprim resistance (Tmp^R)) which is 40% cotransducible with *dnaA*, was P1 transduced from TP91 into the *dnaA46* strain NL19. A Tmp^R, temperature-sensitive clone (NL191) was purified and a P1 lysate prepared. This lysate was used to transduce both NL192Ω pIRF1 and pHCF3 to Tmp^R and the resulting clones were tested for their temperature sensitivity (Table 5.8.1).

Table 5.8.1 Transduction of NL192Ωs to Tmp^R with P1 *dnaA46*, ::Tn*7*.

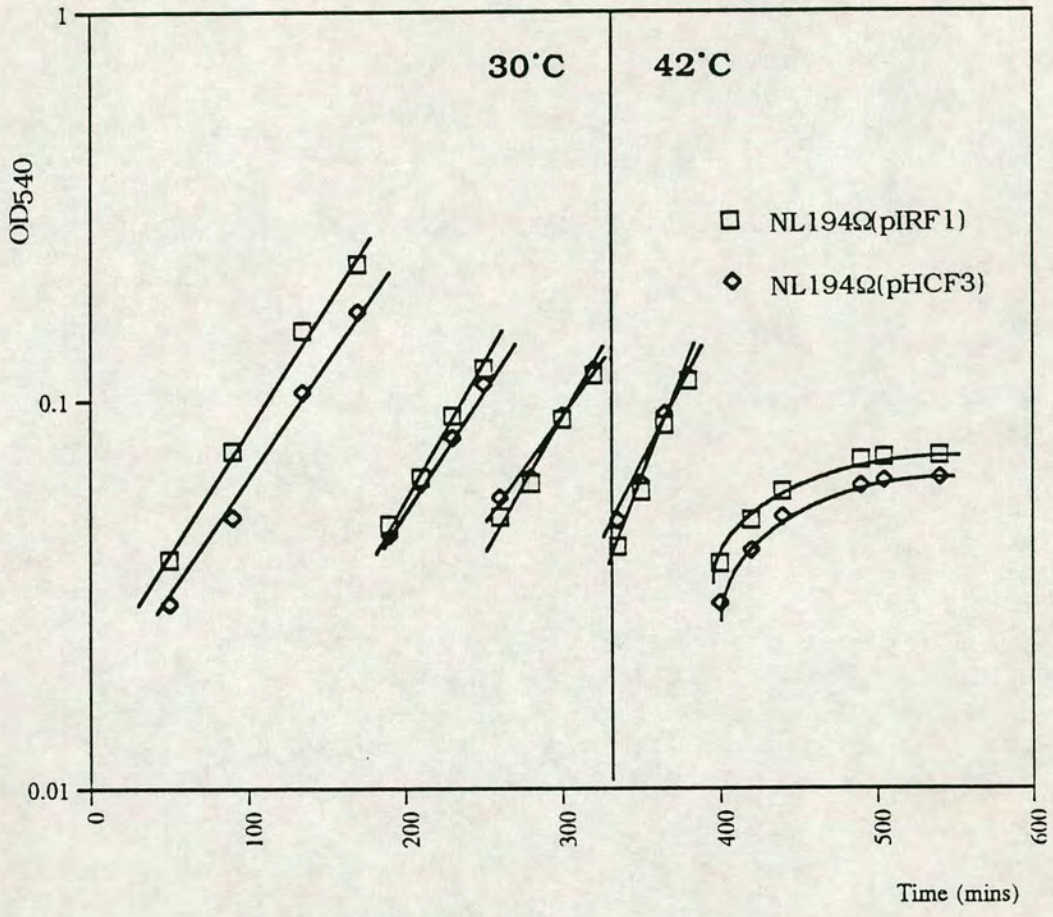
Strain	No. of Tmp^R colonies	No. of Tmp^R/temp sensitive colonies	Cotransduction freq. (%)
NL192Ω pIRF1	171	65	38
NL192Ω pHCF3	203	85	42

Strains were transduced with a P1 lysate from NL191 (*dnaA46*, ::Tn*7*) selecting for Tmp^R. Transductants were then patched onto plates at 30 and 42°C and indubated overnight. Patch growth was then scored accordingly.

As can be seen little difference was observed in the proportion of temperature-sensitive clones recovered from the two strains. However, upon restreaking it was found that the strain bearing the wild-type copy of the *groEL* gene seemed to grow slightly better than that carrying the truncated one. Growth curves showed that NL194Ω

(*groEΔ*::Ω, *dnaA46*, ::Tn7) pIRF1 did grow marginally faster than NL194Ω(pHCF3) but both showed similar temperature sensitivity at 42°C (Figure 5.8.1). The difference in growth rates (as judged by colony sizes) was more obvious on the plates.

Figure 5.8.1 Growth curves of NL194Ω strains with 30–42°C temperature shift.



Growth curves were performed in L-broth as before (see *5.3.1*)
Doubling times NL192Ω(pIRF1), 30°C = 45 ± 4 min.
NL192Ω(pHCF3), 30°C = 49 ± 4 min.

It can be seen that even though *groEL*$_{tr}$ cannot suppress the *dnaA46* mutation, cells that produce DnaA46 and only the truncated GroEL protein are quite viable and not seriously debilitated, in comparison

with those cells which carry *dnaA46*, *groEL*$^+$. This might suggest that DnaA protein maturation does not utilize GroE proteins at lower temperatures although there is evidence against this notion since cold sensitivity (owing to overinitiation of DNA replication from *oriC*) occurs when *groE*$^+$ is overexpressed in suppressed *dnaA*^{ts} strains. I believe, however, that the slight difference in growth rate that was repeatably observed is an indication that any association between GroEL$_{tr}$ and DnaA46 is not as proficient as that between GroEL$^+$ and DnaA46.

5.9 Competition experiments

The above work had involved looking for a phenotypic difference between strains producing the wild-type GroEL protein as opposed to GroEL$_{tr}$. Collaborative work performed by Alexander Girshovich in the (then) USSR had shown that there seemed to be very little difference between the wild-type and truncated GroEL's when subjected to *in vitro* analysis (see *Chapter 5.10* for more details). The greatest difference found was that the truncated GroEL hydrolysed ATP 1.5 times more slowly than did the wild-type protein. A 1.5-fold difference is small and might not be expected to result in discernible phenotypic effects; however when the cell requires a very large amount of active GroE protein, as in the case of suppression of *dnaA*^{ts} mutations, GroEL activity may become rate limiting. This might be manifested as an inability to suppress *dnaA*^{ts}. If this were the case then in other situations that require more GroE function (such as high-temperature growth) the cells with the truncated GroEL may be slightly worse off than are their wild-type counterparts. To test this a competition experiment was devised to detect possible small differences in growth rates between the strains. During the construction of the Tmp^R, *dnaA46* NL192Ω varients (see above), Tmp^R, *dnaA*$^+$ clones were also isolated. This meant that two sets of strains carrying *groEΔ*::Ω with either pIRF1 (*groEL*$^+$) or pHCF3 (*groEL*$_{tr}$) were available, one of which was Tmp^R and the other Tmp^S. The Tmp^R strains were designated NL193Ω. The experiment involved mixing together equal volumes of NL192Ω(pIRF1) and NL193Ω(pHCF3) cultures which had been grown separately at 30°C. Mixes were diluted 10^{-6}-fold into L-broth, split in half and incubated at either 30 or 42°C. The reciprocal experiment was also performed by mixing together NL193Ω pIRF1 and NL192Ω pHCF3 and treating in the same way. An aliquot from each flask was plated out on agar with no trimethoprim and incubated at 30°C overnight. The plate was then replicated onto CAA plates either with or without trimethoprim. This gave a measure of the Tmp^R:Tmp^S ratio. The cells from the overnight incubated flasks were diluted down 10^{-6}-fold into fresh L-broth and the Tmp^R:Tmp^S ratio determined as before. If one strain was growing only slightly better than the other then its dominance in the culture would become amplified by this

technique and an increasing difference in the Tmp^R:Tmp^S ratio would be seen. The results from this experiment were quite striking (Table 5.9.1).

Table 5.9.1 Competition experiments.

A	**0**			**24**			**48**			**72 hrs**		
Temp	Total	Tmp^R	Ratio	Total	Tmp^R	Ratio	Total	Tmp^R	Ratio	Total	Tmp^R	Ratio
30°C	130	63	1.1	172	82	1.1	111	39	1.9	160	56	1.9
42°C				100	12	7.3	138	18	6.7	139	3	45

B	**0**			**24**			**48**			**72 hrs**		
Temp	Total	Tmp^R	Ratio	Total	Tmp^R	Ratio	Total	Tmp^R	Ratio	Total	Tmp^R	Ratio
30°C	50	23	0.9	128	76	1.5	134	90	2.1	132	91	2.2
42°C				100	84	5.3	135	121	8.7	146	145	145

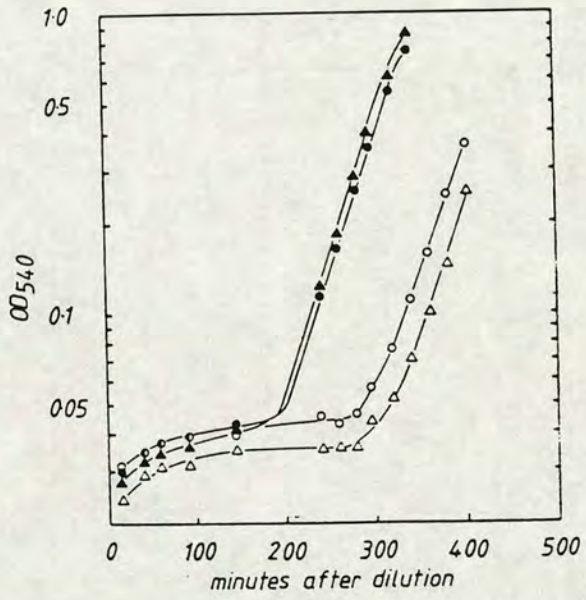
A. NL192Ω pIRF1 (Tmp^S) + NL193Ω pHCF3 (Tmp^R)
B. NL192Ω pHCF3 (Tmp^S) + NL193Ω pIRF1 (Tmp^R)
Ratio expressed as pIRF1-containing cells/pHCF3-containing cells.
See text for experimental details.

At 30°C the cells with the wild-type GroEL protein grew slightly better than the GroEL$_{tr}$ carriers, but at 42°C this difference was much greater; after three cycles of transfer wild-type cells outnumbered the mutants by 100-fold, thus the GM tail of the GroEL protein does indeed confer advantages to the cell under the conditions of this experiment.

In order to find out why the cells carrying pHCF3 fared worse than those with pIRF1, a comparison was made between 48 hour cultures of NL192Ω and NL193Ω maintained at 42°C. Since growth rates during exponential phase were found to be the same for both cultures other phases of growth were examined. This analysis was done by M. Masters and Y. Charters in our laboratory. The maximum optical densities of the cultures attained during stationary phase were

found to be the same, suggesting that the numbers of cells present were similar, and the viability of cells even after prolonged stationary phase incubation was found to be the same for both strains, showing that the strain carrying GroEL$_{tr}$ survives stationary phase as well as does that carrying the wild-type protein. However, it was found that cells carrying pHCF3 exhibit a longer lag before division during exit from stationary phase compared to wild-type cells. That is, the lag phase is extended even though the finally achieved log-phase growth rate is the same in both cases (Figure 5.9.1).

Figure 5.9.1 Recovery of NL192Ω strains from stationary phase.



L-broth cultures were inoculated with 10^{-4} cells ml^{-1} and incubated with shaking for 48 hours, diluted 100x into fresh L-broth and the OD_{540} followed and plotted against time. ▲, NL193Ω(pIRF1); ●, NL192Ω(pIRF1); Δ, NL193Ω(pHCF3); o, NL192Ω(pHCF3). This experiment was performed by M. Masters and Y. Charters.

Stationary phase in *E. coli* is a discrete part of the growth cycle, with expression of a characteristic set of proteins (Groat *et al.*, 1986) that are known to include GroE proteins and other HSPs (Jenkins *et al.*, 1991). Cells entering stationary phase have to make many changes to their molecular anatomy in order to survive stationary phase and allow recovery on the addition of nutrients (Siegele and Kolter, 1992). It is therefore possible that the GroE proteins could be involved with

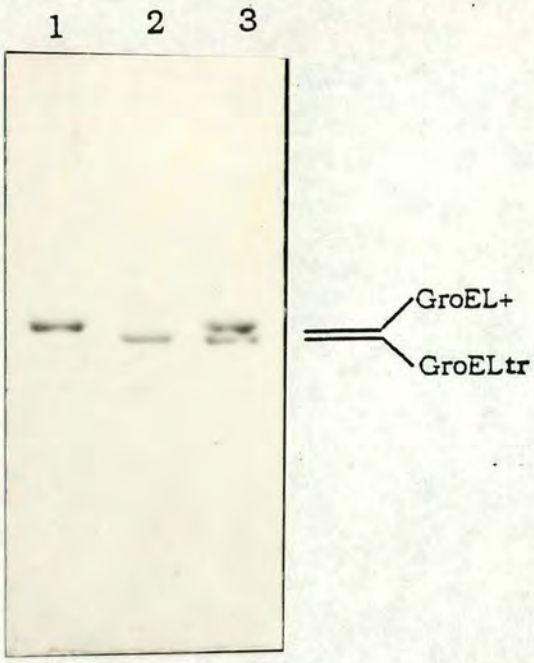
the rapid molecular-assembly requirements of the cell upon the switch from stationary to growth phase. It seems that the GM tail of GroEL is involved in this process, although under laboratory conditions cells carrying GroEL$_{tr}$ were only marginally compromised compared to controls.

5.10 *In vitro* analysis of GroEL$_{tr}$

In the course of this work, a collaboration was set up with A. Girshovich, initially at the Institute of Protein Research (in the then USSR), with the aim of biochemically analysing GroEL$_{tr}$ *in vitro*. It must be clearly stated that my own part in this work was only to supply Girshovich's laboratory with plasmid pHC18, but since this work is relevant to the study reported here, I think it worthwhile to summarise it at this point. The analysis itself was performed by N.M. Lissin.

A preparation of GroEL$_{tr}$ was purified from NL13 (*recA56*, *groE*$^+$) carrying pHC18 in the native 20S tetradecameric form (Bochkareva *et al.*, 1992). Wild-type GroEL was purified from HB101 (pGroE4). Figure 5.10.1 shows wild-type, truncated and mixed forms of the proteins on SDS–PAGE. Even though GroEL$_{tr}$ was purified from a strain which carries the wild-type gene at the chromosomal locus, the gel shows that the wild-type protein does not form a significant part of the preparation. This suggests that the plasmid copy-number of pHC18 is high and proves that the truncated form of the protein can assemble into oligomers which probably do not need to be hetero-oligomers.

Figure 5.10.1 SDS–PAGE of purified GroEL$^+$ and GroEL$_{tr}$.



1 GroEL$^+$
2 GroEL$_{tr}$
3 GroEL$_{tr}$ +GroEL$^+$

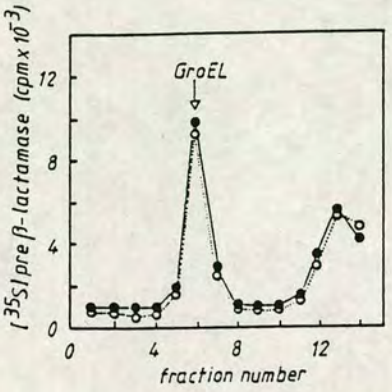
GroEL$^+$ tetradecamers were isolated from HB101(pGroE4), GroEL$_{tr}$ tetradecamers were isolated from NL13(pHC18). Purification was performed by N.M. Lissin.

The stabilities of the oligomeric forms of the wild-type and truncated GroELs were compared in two ways (Lissin *et al.*, 1990). Firstly, in 3 M urea at 0°C it was found that the wild-type dissociated into monomers at around 46%, and the truncated at 40%. Secondly the ellipticities of the two proteins were measured as a function of temperature at 222 nm using circular dichroism. Here both wild-type and truncated forms showed identical melting curves with transition points at 67°C (data not shown). This shows that, at least *in vitro*, the stabilities of the wild-type and truncated forms of the protein are very similar.

Certain biochemical activities of both forms of GroEL were measured. GroEL has been shown to bind to pre-β-lactamase, and

facilitate its translocation into inverted membrane vesicles (Bochkareva *et al.*, 1988). Here, both forms of the protein were found to bind newly formed pre-β-lactamase equally well *in vitro* (Figure 5.10.2).

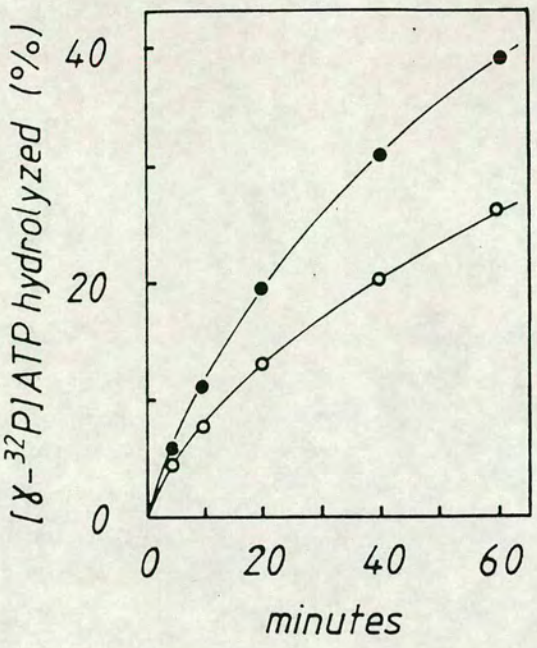
Figure 5.10.2 Interaction of pre-β-lactamase with GroEL/GroEL$_{tr}$.



Post-translational interaction of newly synthesized [^{35}S]-pre-β-lactamase with 1 μM GroEL^{+} (●) or GroEL$_{tr}$ (o). Reaction mixes were analysed by ultra-centrifugation in a sucrose gradient (Bochkareva *et al.*. 1988). Assays were performed by N.M. Lissin.

A membrane translocation assay was not performed with GroEL$_{tr}$, but the fact that GroEL$_{tr}$ does not seem to inhibit β-lactamase excretion *in vivo*, and that even temperature-sensitive *groEL*-carrying strains are not completely compromised for this activity suggested that results comparable to that found with wild-type GroEL would be found. GroEL's ATPase activity is partially blocked upon interaction with GroES (Chandrasekhar *et al.*, 1986; Viitanen *et al.*, 1990). GroES was found to inhibit the ATPase activities of both wild-type and truncated GroEL equally well (data not shown). However, a difference was found in the rates of ATP hydrolysis for the two forms of the protein (Figure 5.10.3); GroEL$_{tr}$ hydrolyses ATP 1.5 times more slowly that the wild-type protein.

Figure 5.10.3 Hydrolysis of ATP by GroEL$^+$ and GroEL$_{tr}$.



GroEL$_{tr}$ (o) hydrolyses [γ^{32}P]-ATP more slowly than does GroEL$^+$ (●) at 25°C. The concentration of GroEL and of [γ^{32}P]-ATP are 0.036 μM and 0.1 mM respectively. These ATPase assays were performed by N.M. Lissin.

Although this is only a slight difference it can be used to explain some of the *in vivo* characteristics seen with strains carrying the GroEL$_{tr}$ protein.

5.11 Summary and Discussion

Phenotypic differences between strains carrying *groE*Δ::Ω plus the plasmids pIRF1 (GroEL$^+$) or pHCF3 (GroEL$_{tr}$) were sought, mainly by comparing their abilities to grow under different environmental conditions. Temperature and carbon source variations were tested and no differences could be found between the strains. *GroEL* mutants are unable to propagate certain bacteriophages and growth of some of these were tested on the two strains; again no differences were observed. SOS repair mechanisms are thought to require the GroE proteins but no differences were found when comparing the UV irradiation resistances of the two strains even though *groE* mutants were found to be defective. Translocation of β-lactamase from cytoplasm to periplasmic space is known to require the function of the GroE proteins. However translocation of β-lactamase was apparently unaffected in strains carrying GroEL$_{tr}$ relative to GroEL$^+$. It can be said, therefore, that the GM tail of GroEL does not greatly influence any of these processes and that mutations of *groEL* that are deficient in any of these processes are unlikely to be found in this region. Indeed DNA sequence analysis on *groEL* mutants deficient in bacteriophage propagation has revealed that none of these mutations is found in or near the DNA encoding the GM motif (Zeilstra-Ryalls *et al.*, 1991a).

Overexpression of the truncated GroEL from high-copy number plasmids does not suppress certain *dnaA*^{ts} mutations, or cause the cold sensitivity associated with suppression by GroEL. *GroEL*$_{tr}$ *dnaA46* strains were found to grow marginally slower than *groEL*$^+$ *dnaA46* at permissive temperatures but the maximum growth temperature of the two strains was the same. This result may indicate that the DnaA46 protein (and therefore possibly the DnaA$^+$ protein) is a true *in vivo* substrate for the GroE proteins. It is quite possible that the GroEL$_{tr}$ is less efficient than its wild-type counterpart for some biological processes (see below) and this reduction in efficiency may be amplified by the presence of a mutant form of DnaA, leading to the differences in growth rate observed. This difference could be too subtle to be noticed in *dnaA*$^+$ strains. A closer analysis of these strains, such

as measuring DNA synthesis, may prove informative and will hopefully be included in future investigations.

The only difference found between the two GroEL proteins *in vitro* was that the truncated hydrolyses ATP 1.5 times slower than the wild-type. The ATPase activity of GroEL is believed to be involved with the release of some bound polypeptides. This difference in the rates of ATP hydrolysis is not large but does suggest that *in vivo* effects, especially in circumstances requiring higher levels of GroEL activity may be apparent. Indeed, the competition experiments in which the cells expressing *groEL*$^+$ and *groEL*$_{tr}$ were grown together over many generations showed that the wild-type protein does confer an advantage over the truncated under certain conditions, particularly under those when the cell's GroE requirement is presumably high, such as continuous growth at 42°C. Closer analysis demonstrated that the cells carrying the truncated version of GroEL were slower in their recovery from stationary phase; the lag phase in these cells being greater by 20 minutes or more than that of its wild-type counterpart when diluted from a 48 hour culture at 42°C into fresh, rich medium.

The *in vitro* studies showed that the stabilities of GroEL tetradecamers made up of wild-type or truncated proteins are the same and SDS–PAGE analysis suggests that the two forms of the proteins are maintained at similar levels. It seems reasonable therefore that the differences seen between strains carrying *groEL*$^+$ and *groEL*$_{tr}$ are caused by a slightly reduced activity (possibly related to the reduction in ATPase activity) associated with the truncated protein. It also seems that this discrepancy manifests itself more fully at heat-shock-inducing temperatures. It makes sense for the cell to require more GroEL activity at 42°C than at 30°C, and so differences would become amplified (as is seen in the competition experiment). This reduction of GroEL activity could also be used to explain the lack of *dnaA* suppression by overexpression of *groEL*$_{tr}$. For *groE* to suppress *dnaA* the proteins must be present in very large quantities. Again a small reduction in GroEL activity could be important in a case in which so much protein is required to perform a specific task, such as is seen in *dnaA* suppression. However, if the GroEL$_{tr}$/DnaA46 interaction is only less efficient than that of GroEL$^+$/DnaA46 then one might expect *dnaA46* strains carrying *groEL*$_{tr}$ on a high-copy-number

plasmid to show intermediate suppression at temperatures approaching 42°C and some degree of cold sensitivity at 30°C. Since neither of these effects are seen with *dnaA46*, it is possible that the GM motif of GroEL could have a specific role in the suppression of *dnaA*^{ts}.

5.12 Further studies on *groEL*$_{tr}$

After completion, and submission for publication, of the aforementioned work (McLennan *et al.*, 1993) it came to our attention that another research group studying the GroEL protein had also found that the GM tail of GroEL is dispensible for growth (A. Horwich, personal communication). However, they found a phenotypic difference between strains expressing wild-type or truncated versions of GroEL. Strains depending upon GroEL$_{tr}$ were unable to grow on minimal agar supplemented with L-rhamnose as sole carbon source. The details of their experiments are not known. L-rhamnose is a six carbon methylpentose sugar and is dissimilated by enzymes of the *rha* operon (consisting of a permease, kinase, isomerase, aldolase and an activator protein) located at 87.7 minutes on the genetic map (Baldoma and Aguilar, 1988; Lin, 1987). On hearing of this it was decided to test our strains for the ability to utilize L-rhamnose. In addition to minimal agar supplemented with either glucose or L-rhamnose, MacConkey agar plates containing glucose or L-rhamnose were used. MacConkey agar is an indicator medium used to demonstrate the ability of a strain of *E. coli* to utilize the sugar present. If a sugar is utilized then the resulting colonies on the plate will appear red, if not then the colonies are white.

NL192Ω(pIRF1) and NL192Ω(pHCF3) were streaked out on supplemented minimal agar containing 0.2% glucose or L-rhamnose, and on MacConkey agar plates containing 1% glucose or L-rhamnose. The plates were incubated overnight at both 30 and 42°C. Examination of the plates proved interesting. As expected in the case of the minimal agar both strains grew equally well on plates containing glucose at both temperatures. However, with L-rhamnose as the carbon source NL192Ω(pHCF3) (GroEL$_{tr}$) did not grow at 42°C although growth at 30°C was similar to that seen with NL192Ω(pIRF1) (GroEL$^+$) (Table 5.12.1).

Table 5.12.1 Growth on L-rhamnose/glucose-containing minimal agar.

	Minimal + glucose		Minimal + L-rhamnose	
Strain	**30°C**	**42**	**30**	**42**
NL192Ω pIRF1	++	+++	++	++
pHCF3	++	+++	++	-[a]

Strains were streaked onto VB minimal agar (with the required amino acid supplements) plus either glucose or rhamnose and incubated at 30 and 42°C until colonies had formed. +++, well formed colonies/-, no colonies visible.
a. Sometimes tiny colonies were seen on these plates.

This result was therefore consistent with the results that had been communicated to us, although we do not know whether Horwich had found L-rhamnose utilization to be temperature dependent. This is an interesting result and suggests both that rhamnose utilization requires GroEL protein function and that this function requires the presence of the GM-tail in the GroEL protein (at least at 42°C). The L-rhamnose metabolic pathway converges with that of L-fucose, another methylpentose sugar (Baldoma and Aguilar, 1988). It was found that my strains carrying GroEL$_{tr}$ were able to grow on L-fucose both at 30 and 42°C (data not shown). This suggests that the block in L-rhamnose dissimilation occurs before the L-rhamnose and L-fucose pathways converge. It is, therefore, one of the *rha* operon enzymes that is the candidate for GroEL interaction and not L-lactaldehyde dehydrogenase (the product of the *ald* locus) which is required for aerobic utilization of both L-rhamnose and L-fucose (Chen *et al.*, 1987; Baldoma and Aguilar, 1988). The intriguing result that strains carrying GroEL$_{tr}$ are temperature sensitive for growth on rhamnose is certainly worthy of further investigation (even though I did not make the original observation). Our result was both clear-cut and reproducible. The results from the MacConkey plates were, however, more surprising (Table 5.12.2).

Table 5.12.2 Growth on L-rhamnose/glucose-containing MacConkey agar.

Strain	MacConkey + glucose		MacConkey +L-rhamnose	
	30°C	42	30	42
NL192Ω pIRF1	+++	+++	+++	+++
pHCF3	+++	+[a]	+++	-

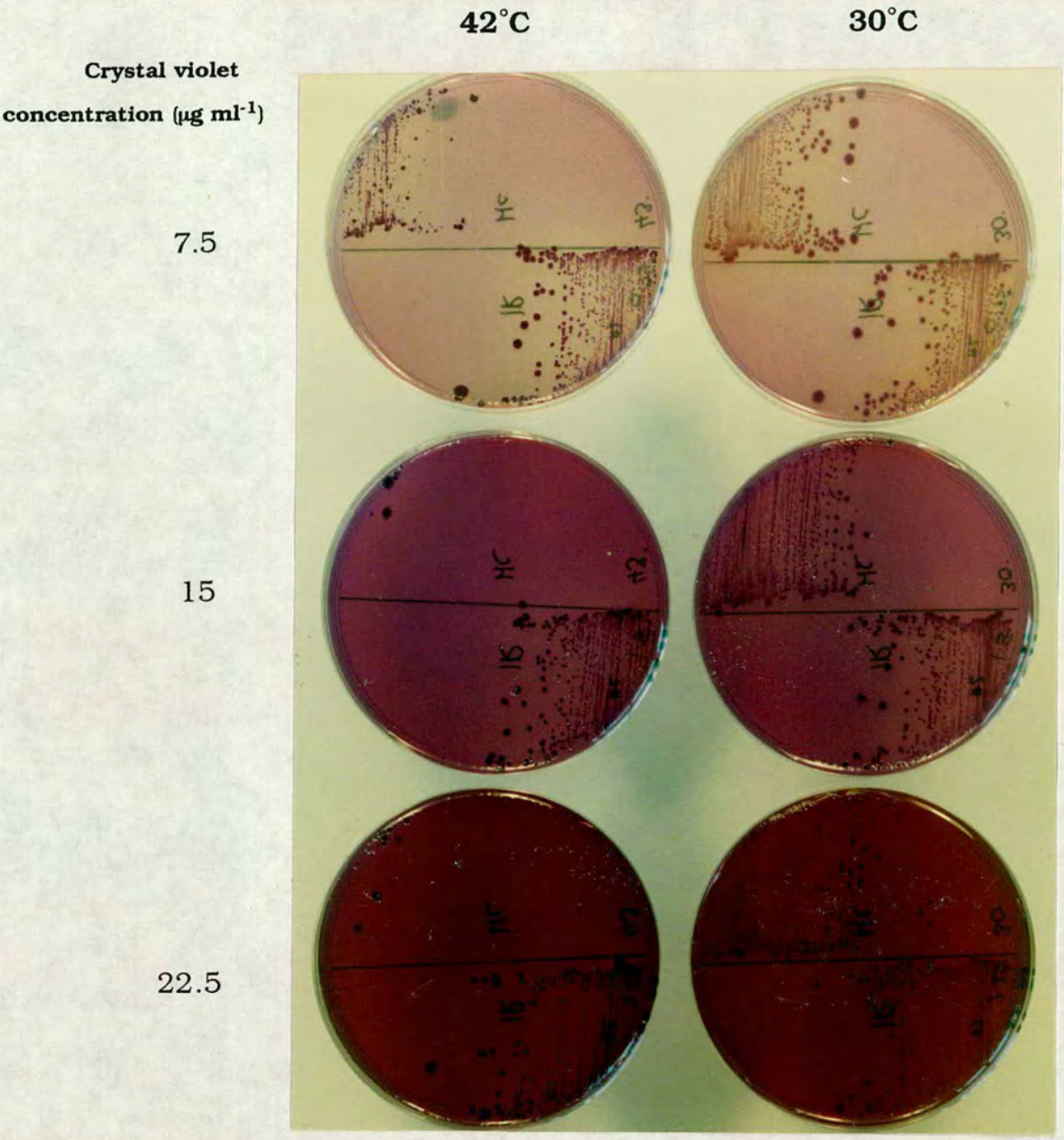
Strains were streaked onto MacConkey agar plus either glucose or rhamnose and incubated at 30 and 42°C overnight. +++, well formed colonies/-, no colonies visible. a. Colonies greatly reduced in numbers as well as size.

In all cases NL192Ω(pIRF1) behaved as expected with good growth and red colonies. With NL192Ω(pHCF3) growth was normal at 30°C, but temperature sensitivity at 42°C was seen on both the glucose-containing and L-rhamnose-containing MacConkey agars, with the growth seen on the former slightly better than that on the latter. This was unexpected since *E. coli* strains normally grow on MacConkey agar even if an exogenous sugar carbon source is not available to the bacteria. In fact MacConkey agar is used as a selective medium to distinguish between other enteric bacteria and *E. coli*. It seemed that there was something in the agar inhibiting the growth of strains expressing the truncated GroEL at high temperature. In order to determine what was causing this effect the components of MacConkey medium were analysed individually.

The most obvious candidate constituent was bile salts. It is the bile salts in MacConkey medium that inhibits the growth of non-coliform bacteria. L-agar plates containing either 2 or 5% sodium deoxycholate (bile-salt detergent) were made. NL192Ω(pIRF1) and NL192Ω(pHCF3) were plated on these at 30 and 42°C. After incubation, examination of the plates showed that both strains had coped equally well on both concentrations at both temperatures. This suggested that it was not the bile salts that had caused temperature sensitivity. MacConkey agar contains two dyes, neutral red and crystal violet (also known as gentian violet). L-agar plates containing 25 and 50 μg ml^{-1} neutral red (MacConkey medium contains 30 μg ml^{-1}) were used as above but again no differences were seen between

the two strains. The strains were plated out onto L-agar containing either 5 or10 μg ml^{-1} crystal violet (MacConkey medium contains 1 μg ml^{-1}) at both 30 and 42°C and incubated overnight. It was found that at 30°C both strains grew equally well but at 42°C growth on crystal violet of strains carrying GroEL$_{tr}$ was retarded, with growth on the higher concentration being particularly poor. (See Figure 5.12.1 for an extended example.)

Figure 5.12.1 Growth of NL192Ω pIRF1/pHCF3 on L-agar containing crystal violet at 30 and 42°C.



Strains were streaked out on L-agar containing 7.5, 15, and 22.5 μg ml^{-1} crystal violet and incubated for 48 h at 30 and 42°C. In each case NL192Ω(pHCF3) is on the top half of the plate, streaked from left-to-right, and NL192Ω(pIRF1) is on the bottom half of the plates, streaked from right-to-left. It can be seen that NL192Ω(pHCF3) is temperature sensitive for growth on >7.5 μg ml^{-1} crystal violet.

Buxton *et al.* (1983) suggest that the minimum inhibitory concentration of crystal violet for *E. coli* K-12 is >200 μg ml^{-1} and so the NL192Ω(pHCF3) cells have become extremely sensitive to the dye. Examination of these cells under the microscope showed that filamentation had occurred suggesting that cell division had been blocked in some way. This result demonstrated that it was the dye crystal violet, present in MacConkey medium, which had caused the observed temperature sensitivity of GroEL$_{tr}$ containing strains. This fortuitous discovery gave renewed hope for unearthing a potential role for the GM tail of *E. coli*'s GroEL protein.

The dye crystal violet has long been known to display anti-bacterial activity and had been used ectopically to treat certain infections prior to the widespread availability of modern antibiotics. A 1% solution of crystal violet is included in the World Health Organisation essential drugs list as an anti-infective drug and skin disinfectant (Bakker *et al.*, 1992). The basis of the toxicity of crystal violet and other triphenylmethane dyes has not been established but their ability to bind DNA (Fox *et al.*, 1992) and ribosomes (Gale *et al.*, 1981) *in vitro* may signal a similar action *in vivo*.

There are mutants of *E. coli* that show a reduced tolerance to exposure to certain dyes. Strains carrying the mutations *envA* or *dye* behave in this way (Normark *et al.*, 1969; Normark, 1970; Buxton *et al.*, 1983). These strains are also sensitive to normally sub-lethal doses of several other anti-bacterial agents. *EnvA* mutant bacteria are reduced in their capacity to divide and form chains of cells. It has been proposed that such mutant cells are 'leaky' and their cell envelopes less resistant to penetration by environmental agents. As a result more of the agents enter the cell and toxic levels are reached at lower external concentrations. In the case of *dye* mutants (isolated owing to an increased sensitivity to the dye toluidene blue) it has been shown that they have altered outer and inner membrane protein profiles. Male F or Hfr cells are sterile in the presence of *dye* mutations and the Dye protein is believed to effect the transcription of F-encoded genes (Buxton *et al.*, 1983). It is likely that Dye is also involved with transcription of certain membrane-associated protein genes and it seems that this causes their increased permeability to normally sub-lethal doses of certain antibiotics.

It was therefore decided to test the resistance of our strains to agents that specifically affect *envA* or *dye* mutants in order to see whether these proteins are likely to be adversely affected in cells expressing *groEL*$_{tr}$. NL192Ω(pIRF1) and (pHCF3) were streaked on L-agar plates containing various concentrations of several antibacterial agents and other dyes and incubated overnight at both 30 and 42°C. The results are shown in Table 5.12.3. Out of all the agents tested only crystal violet, nalidixic acid and ampicillin demonstrated an effect. This suggested that the effects seen with *groEL*$_{tr}$ are different to those observed with *envA* and *dye* mutants.

Table 5.12.3 MIC of NL192Ω strains on various biocides at 30 and 42°C.

Strain	Amp		CV		Cmp		Nal		TB		MB		SDS		NaDe	
	30	42	30	42	30	42	30	42	30	42	30	42	30	42	30	42
NL192Ω																
pIRF1	3	4	>30	>30	2	2	4	11	>50	>50	>50	>50	>8*	>8	>4*	>4
pHCF3	3	3	>30	10	2	2	4	7	>50	>50	>50	>50	>8	>8	>4	>4

Strains were plated out on medium containing a range of concentrations of various chemicals and incubated at 30 and 42°C. Values given are the minimum inhibitory concentration (MIC) as judged by these experiments. Values are given in μg ml^{-1}, except SDS and NaDe (*) where the values are given as a percentage. Amp, ampicillin; CV, crystal violet; Cmp, chloramphenicol; Nal, nalidixic acid; TB, toluidene blue; MB, methyl blue; SDS, sodium dodecyl sulphate; NaDe, sodium deoxycholate.

It was also found that NL192(pHCF3), a strain which is diploid, carrying single copies of both wild-type and truncated GroEL, could grow well on crystal violet plates at 42°C demonstrating that the effect seen with GroEL$_{tr}$ is recessive.

Another dye with antibacterial activity and a similar chemical structure to crystal violet is malachite green (also known as brilliant green). The presence of malachite green in L-agar plates at a concentration of 10 μg ml^{-1} resulted in NL192Ω(pHCF3) growing slightly slower than the strain carrying pIRF1 when incubated at the higher growth temperature of 42°C, although the differences were not as impressive as they were with crystal violet-containing medium. As

with crystal violet, no significant differences were seen between the two strains at 30°C.

Summary and discussion

Previous work described in this chapter demonstrated that although differences between strains carrying either *groEL*$^+$ or *groEL*$_{tr}$ were hard to detect, they were found. A fortuitous discovery, made just as this particular line of investigation was being completed, has uncovered a clear requirement for the carboxyl-terminal GM motif of GroEL for dye resistance at higher temperatures. It has been found that strains producing GroEL$_{tr}$ are hypersensitive to the dye crystal violet when incubated at 42°C. Normally *E. coli* is extremely resistant to penetration by crystal violet since it is hydrophobic and cannot traverse the outer membrane (Gustafsson *et al.*, 1973). Obviously if crystal violet is killing the cell then it must be penetrating the membrane in some way. Disruption of the outer membrane lipopolysaccharide can allow penetration by hydrophobic molecules but such cells are also hypersensitive to bile salts and other detergents such as SDS. Cells carrying GroEL$_{tr}$ are not affected by such agents. Other mutants, for example *envA* and *dye* are also sensitive to normally sublethal doses of certain chemicals and this is thought to be caused by an incorrect membrane composition. The *dye* mutants show remarkable specificity towards certain environmental agents, and there is no correlation between toxicity and hydrophobicity of the compounds tested (Buxton *et al.*, 1983). This is also true for strains with GroEL$_{tr}$ since sensitivity is seen to crystal violet (hydrophobic) and ampicillin (hydrophilic). However *dye* mutants are only slightly more sensitive to crystal violet than *dye*$^+$ cells and cells carrying GroEL$_{tr}$ are not sensitive to toluidine blue, the dye to which *dye* mutants are sensitive. Similarly the effects seen with GroEL$_{tr}$ are different to those observed with the *envA* mutation since although these cells are hypersensitive to ampicillin, nalidixic acid and crystal violet (as are those carrying GroEL$_{tr}$) they are also hypersensitive to chloramphenicol and sodium deoxycholate while cells depending on GroEL$_{tr}$ are not.

From these results it seems that cells carrying GroEL$_{tr}$ are different from their isogenic wild-type counterparts in that they can be

penetrated by certain toxic environmental factors at 42°C (but not at 30°C). The spectrum of chemicals that do have an influence on the viability of cells with GroEL$_{tr}$ suggests that this effect is different to that caused by mutations in either *dye* or *envA*. Further investigations examining membrane preparations and analysing suppressing mutations may prove useful in determining in what ways these cells physiologically differ from the wild-type. (Note that re-examination of the SDS–PAGE of total membranes preparations from these cells (see 5.7) shows no obvious major differences between the protein profiles from these cells, but this is far from definitive.)

CHAPTER 6

FURTHER TRUNCATION OF THE *GROEL* GENE

6.1 Introduction

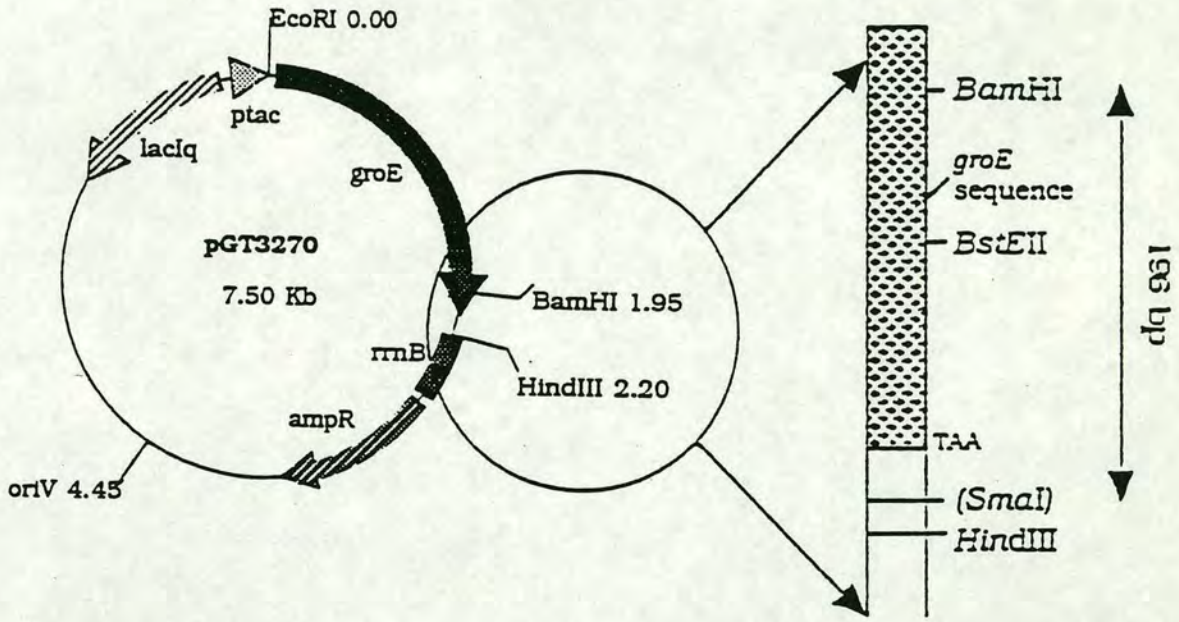
The results in *Chapter 5* demonstrate that the highly conserved 18 amino acid GM tail of GroEL is dispensable for normal function, and I have proposed that the slightly reduced ATPase activity exhibited by GM-lacking GroEL$_{tr}$ *in vitro* is a possible cause of the differences that are seen *in vivo*. As a conclusion to this work it seemed reasonable to determine how much of the carboxyl-terminus of the protein could be removed before loss of function was observed. Previous work has shown that truncation of *groEL* up to the *Bam*HI restriction site (removing DNA encoding 53 amino acids) with concomitant addition of DNA encoding ~100 amino acids, resulting in out-of-frame expression of DNA encoding the tetracycline-resistance gene of pBR325, produced a protein that was unable to complement *groEL*^{ts} mutants for high-temperature growth (March, 1988). It was thought possible that the additional amino acids might be rendering this fusion protein non-functional. However, another group have made a GroEL' truncated at the *Bam*HI site without additional non-GroEL amino acids and it too is non-functional (K. Tilly, personal communication).

In order to make further truncations of the GroEL protein, a site-directed mutagenesis (SDM) procedure was adopted. It was decided to create in-frame TAA 'STOP' codons within the GroEL sequence. TAA is the stop codon naturally present at the end of the wild-type gene and so this seemed the obvious choice. To maximize the efficiency of the SDM, single base-pair mutations were used so that the hybridization potential of the mutagenic oligonucleotides would be as high as possible. Bacteriophage M13 was used to provide ssDNA as a template for the SDM.

6.2 Cloning the *groE* operon into bacteriophage M13

The M13 cloning vector chosen for cloning of the *groE* sequence was M13mp18. This phage has a polylinker with restriction sites in the order 5'-*Eco*RI–*Bam*HI–*Hin*dIII-3'. The original plan was to clone as small-a-piece of DNA from the *groEL* region as was possible, as this would minimize the chance of picking up unwanted mutations generated by the *in vitro* replication of the phage DNA. The 200 bp *Bam*HI–*Hin*dIII DNA fragment from pGT3270 containing the terminal 164 bp of the *groEL* gene seemed ideal (Figure 6.2.1).

Figure 6.2.1 The *Bam*HI–*Hin*dIII fragment from pGT3270.

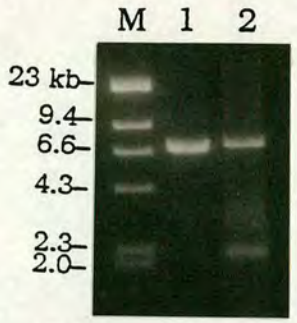


The ~210 bp *Bam*HI–*Hin*dIII fragment from pGT3270 which was intended to be cloned into M13mp18 as a template for site-directed mutagenesis. Note the *Bst*EII site within this fragment that was used to identify M13 clones containing the fragment.

This region also contains a *Bst*EII restriction site not present in the original M13mp18 DNA, and so this, along with the blue/white plaque selection system of the original phage, could be used to easily verify that the intended DNA fragment had been cloned. pGT3270 and M13mp18 DNA were restricted with *Bam*HI and *Hin*dIII. The products

of the restriction were pooled and ligated together. The ligation mix was then transformed into TG1 and plated out in BBL top agar with IPTG and X-gal and incubated overnight. The white plaques were picked and the phage DNA analysed by restriction with *Bgl*II and *Bst*EII, which results in a 7.25 kb *Bgl*II M13mp18 DNA fragment being cleaved by restriction at the introduced *Bst*EII restriction site plus the ~210 bp insert to yield fragments of sizes 6.64 kb and 800 bp. Of the initial 12 plaques analysed, four gave the predicted restriction pattern on an agarose gel and of these one was designated M13EL1 (data not shown). In order to verify absolutely that the correct sequence was present, ssDNA of M13EL1 was prepared and sequenced by the dideoxy chain termination sequencing method using the M13 'universal primer'. This gave unexpected results. The correct DNA fragment had been cloned but it was in the wrong orientation. An initial explanation was that I had accidently cloned the fragment into M13mp19, which has its polylinker in the opposite orientation to M13mp18. Commercially produced M13mp18 dsDNA was obtained and the same cloning attempted again. This time the whole *groE* operon from pGT3270 on a 2.1 kb *Eco*RI–*Hin*dIII fragment was also ligated to *Eco*RI/*Hin*dIII restricted M13mp18 DNA. Of 12 white plaques obtained from the repeat cloning only two gave the expected restiction pattern with *Bst*EII and *Bgl*II, whereas <u>all</u> of the plaques analysed from the cloning of the whole operon had the predicted restriction pattern, i.e. restriction with *Eco*RI and *Hin*dIII generates a 2.1 kb fragment along with the 7.2 kb M13 backbone (Figure 6.2.2).

Figure 6.2.2 Restriction of M13mp18 and M13ESL8.



M λ *Hin*dIII markers
1 M13mp18 *Eco*RI/*Hin*dIII
2 M13ESL8 *Eco*RI/*Hin*dIII

Double-stranded M13mp18 and M13ESL8 DNA was restricted with *Eco*RI and *Hin*dIII, electrophoresed through 0.8% agarose and stained with ethidium bromide. Note the 2.1 kb *groE* fragment released from M13ESL8.

One of the positive clones from each ligation was sequenced. The clone containing the whole *groE* operon generated a sequence which was as expected, corresponding to the 3' end of the *groEL* gene, but the recloning of the small *Bam*HI–*Hin*dIII fragment again gave a sequence of the wrong orientation. Closer analysis of the sequence of the correct, whole operon clone (M13ESL8) showed that in the cloning of the *groE* operon to construct pGroESL (Goloubinoff *et al.*, 1989b) (pGT3270's parent) a naturally occuring *Sma*I site 34 bp downstream from the *groEL* translation termination codon had been used to ligate the 3' end of the operon to a linker carrying a distal *Hin*dIII site. Apparently this linker also contained a *Bam*HI site and thus restriction of pGT3270 with *Bam*HI and *Hin*dIII results in release of a *Bam*HI–*Bam*HI fragment. This could only ligate into M13mp18 molecules that had been singly restricted with *Bam*HI during the *Bam*HI–*Hin*dIII double digest. This probably explains the low frequency of recovered recombinants in this experiment. In some ways it was fortunate that the wrong orientation was originally obtained since if the *Bam*HI–*Bam*HI fragment had ligated into M13mp18 in the orientation that had been expected, then recloning of the final mutagenized products back into pGT3270 could have proved

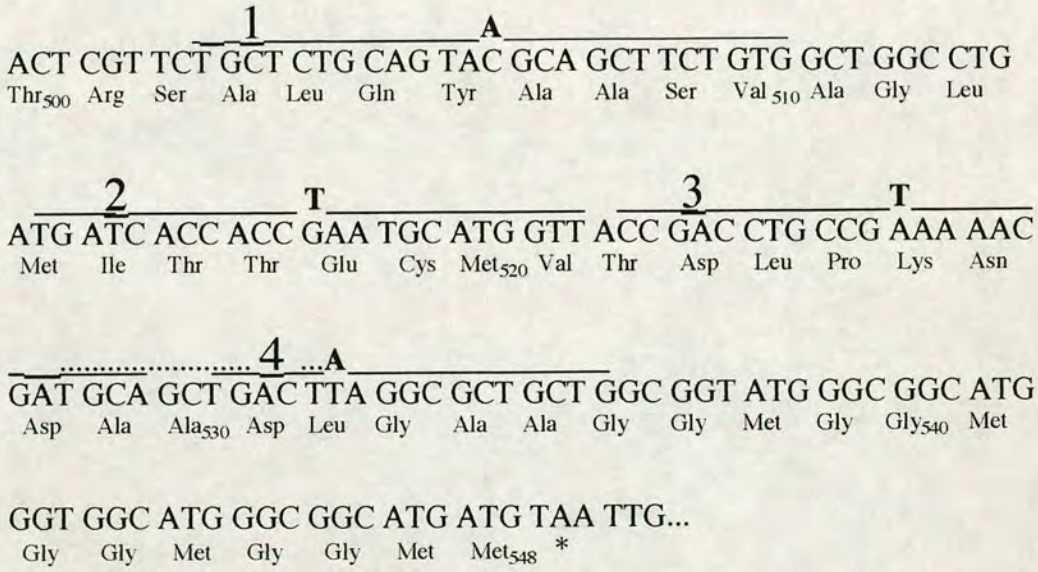
extremely difficult and may have led to the wrong conclusions being drawn. Since M13ESL8 seemed perfectly suitable for the SDM, high quality single-stranded DNA was isolated and this was used for the subsequent reactions.

6.3 Site-directed mutagenesis of the *groEL* gene

The GroEL protein is 548 amino acids long. The original truncated protein constructed in this work contains 531 amino acids (of which 530 are from *groEL*), and the *Bam*HI truncation produces a protein which contains 495 amino acids of GroEL. Oligonucleotides were designed so that the mismatched base was in the centre of an oligonucleotide containing between 21 and 25 bases.

Attempts were made to make four mutations that would result in GroEL' proteins 531, 525, 517 and 505 amino acids long, with the 531 amino acid protein acting as an internal control since its expected behaviour was known (Figure 6.3.1). The SDM procedure used was the method of Taylor *et al.* (1985) manufactured in kit form by Amersham (see the *Materials and Methods* for details).

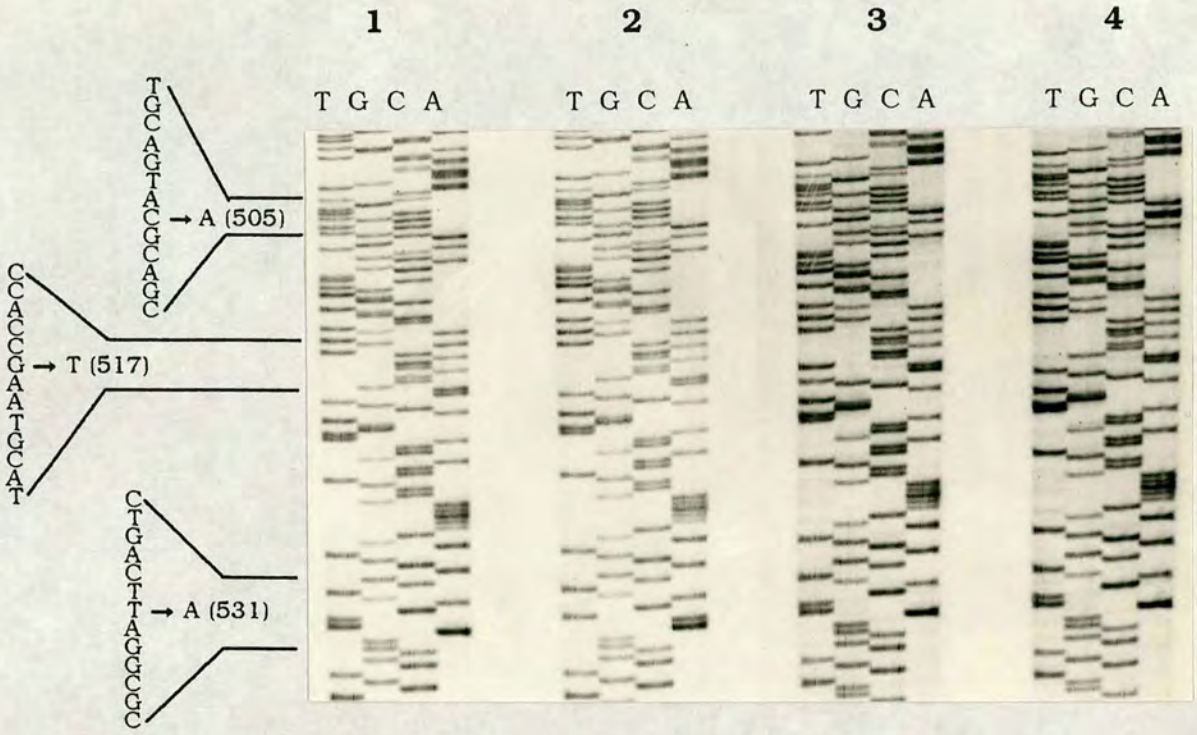
Figure 6.3.1



The 3' end of the *groEL* gene showing the positions of site-directed mutations used to construct in-frame STOP codons. The black lines show the extent of the mutagenic oligonucleotides used (oligos 3 and 4 overlap by four base pairs; the dotted line shows the extent of oligo 4) and the bold letter in each oligo indicates the mismatched base introduced into the coding sequence. All other bases have perfect matches.
Oligo 1 introduces a STOP codon at position 506, producing GroEL505.
Oligo 2 introduces a STOP codon at position 518, producing GroEL517.
Oligo 3 introduces a STOP codon at position 526.
Oligo 4 introduces a STOP codon at position 532, producing GroEL531.
Numbers represent the amino acid number within the GroEL polypeptide.

Oligonucleotides 1, 2 and 4 worked well; after sequencing several clones obtained from each reaction the rate of successful mutagenesis was found to be around 40%. The sequences of the correct constructions are shown in Figure 6.3.2.

Figure 6.3.2 Sequencing gel of M13ESL8-SDM products.



1	M13ESL8–548 (wild-type *groEL* sequence)
2	M13ESL8–531
3	M13ESL8–517
4	M13ESL8–505

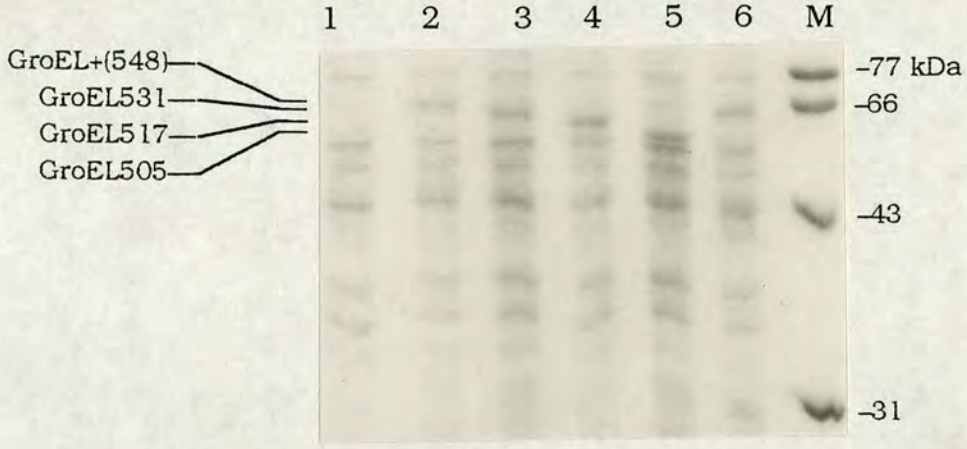
The mutagenized M13ESL8-derivatives were sequenced and electrophoresed through a 15% gel as described in the *Materials and Methods*. The mutations are indicated (→).

However, SDM using oligonucleotide 3 gave no mutant clones. The reason for this is unknown but it is possible that since the mismatch in the oligonucleotide is within a run of adenosine residues it may result in unstable annealing with the ssDNA template. Since three out of the four desired mutagenic products were successfully cloned it was decided to carry on with these, rather than persevere with

oligonucleotide 3. The mutagenized derivatives of M13ESL8 produced using oligonucleotides 1, 2 and 4 were named M13ESL8–505, M13ESL8–517 and M13ESL8–531 respectively.

In order to verify that the mutagenized M13 templates were indeed producing truncated versions of the GroEL protein, TG1 was infected with M13ESL8 and its derivatives 531, 517 and 505. The infected culture was incubated at 37°C for 5 hours in the usual way, and in order to increase expression from the *groE* heat-shock promoter the samples were heat-shocked at 42°C for 30 minutes. These samples were then subjected to SDS–PAGE analysis (Figure 6.3.3).

Figure 6.3.3 SDS–PAGE of total protein from TG1 infected with M13ESL8 derivatives.



1	TG1 infected with M13mp18 whole-cell extract
2	TG1 infected with M13ESL8–548
3	TG1 infected with M13ESL8–531
4	TG1 infected with M13ESL8–517
5	TG1 infected with M13ESL8–505
6	TG1 infected with M13ESL8–548
M	molecular weight markers

TG1 was infected with M13 phage lysates and incubated for six hours at 37°C. The cultures were then heat shocked at 42°C for one hour, and whole-cell extracts prepared and subjected to SDS–PAGE as described in the *Materials and Methods*. The decreasing sizes of the GroEL proteins produced from the phage-infected cells can clearly be seen.

As can be seen, the infected cells (other than those of the control) demonstrate the presence of GroEL proteins that are progressivly smaller with respect to the wild-type protein expressed by M13ESL8. This shows that the isolated mutagenized derivatives of M13ESL8 produce truncated GroEL' proteins that migrate as expected. The next stage in the procedure was to clone the *groE*' inserts into a suitable plasmid vector so that the activity of the protein products could be determined.

6.4 Recloning the SMD products into pJF118

Plasmid pJF118 was chosen as the vector to receive the products of the SDM reactions. In each case double-stranded DNAs from M13ESL8 (548) and each of the mutagenized M13ESL8 clones (producing GroEL505, 517 and 531) were restricted with *Eco*RI and *Hin*dIII. pJF118 DNA was restricted with the same enzymes and the resulting fragments were ligated together. In order to select recombinant plasmids, the ligation mixes were transformed into NL302, a *groES*^{ts} strain, at its non-permissive temperature of 42°C in the presence of ampicillin. This should select for clones that are carrying plasmids containing the complementing wild-type *groES* gene. All the mutagenized M13ESL8 derivatives should still code for a fully functional GroES protein. Initially clones were only obtained from the ligation mixes of M13ESL8–548 and M13ESL8–531, the SDM product of which is very similar to the original GM-lacking truncation that had been made. DNA from positive clones was purified and the correct construction verified by restriction analysis; the truncation was verified by SDS–PAGE analysis (data not shown). The new plasmids were designated pGTR548 and pGTR531.

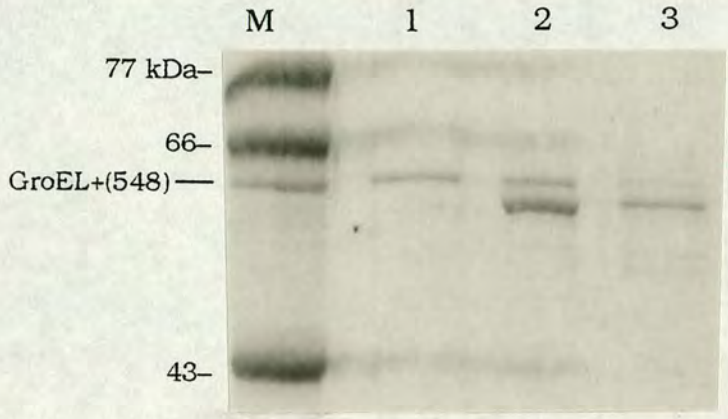
The transformation of NL302 with the ligation mixes containing restricted M13ESL-517 and 505 was repeated. Once again no colonies were obtained with 517, but two were recovered from 505. These were analysed by SDS–PAGE and both were found to be overexpressing a full-length GroEL protein. It was thought that maybe the stock of M13ESL8–505 phage was contaminated with original M13ESL8 phage. The phage stock was repurified from a single plaque and its DNA sequenced. The sequence demonstrated the presence of the intended mutation. The ligation mixes and transformations were repeated again; two further 505 colonies were recovered both of which overexpressed the full-length GroEL protein. The whole procedure had also been carried out several times with the 517 truncation and no viable colonies were ever found. The ligations from 505 and 517 were also transformed more than once into the commonly used cloning strain DH1 but no colonies were recovered. At this point it was thought that perhaps the mutant truncated versions of GroEL were causing biological problems leading to inviability (or a very high

selection pressure to revert) of any cell carrying them. This problem could be in part caused by the high copy number of the plasmid; thus it was thought that a reduction in plasmid copy number might lead to recovery of the desired plasmids. There is a chromosomal gene, *pcnB*, mutations of which lead to a drastic reduction in plasmid copy number, and to plasmid instability (March *et al.*, 1989; He *et al.*, 1993). Plasmids can be maintained in *pcnB* strains by constant selection. Our collection includes strains in which the *pcnB* gene has been replaced with a kanamycin-resistance cassette. This makes transfer of the deletion to other strains relatively easy. A P1 lysate prepared on MM38 *pcnB*Δ::*kan* was used to transduce NL30 (*groES*^{ts}) and NL44 (*groEL*^{ts}) to kanamycin resistance. The ligation mixes (517 and 505) were transformed into NL30 *pcnB*Δ::*kan* (NLP30) at 42°C. This time 505 yielded 60 colonies, but only a single colony of 517 was obtained. Restriction analysis of the resulting DNA showed that in each case a DNA insert of the correct size (2.1 kb) had been obtained. Plasmid DNA from the NLP30 clones was prepared and the resulting plasmids were called pGTR517 and pGTR505. This gave a battery of GroEL' versions with which to attempt *groEL*^{ts} complementation tests; however, it should be noted here that pGTR517 did not encode GroEL517 as had been expected.

The results of the complementation tests can be summarized as follows. The plasmids pGTR548 (GroEL548) and pGTR531 (GroEL531) behaved as expected. They both complemented *groEL*^{ts} mutations for high-temperature growth, and λ phage morphogenesis, but only pGTR548 allowed suppression of *dnaA*^{ts} mutations. Plasmid pGTR505 (GroEL505) did not complement *groEL*^{ts} mutants, and caused very poor growth at 42°C in *groE*$^{+}$, *pcnB*$^{+}$ hosts. The results obtained with pGTR517 were unexpected. The plasmid could not be transformed into *pcnB*$^{+}$ hosts at any temperature, and did not allow complementation of *pcnB*Δ, *groEL*^{ts} mutants. However, purification of GroEL tetradecamers from cells carrying pGTR517 demonstrated the presence of a truncated GroEL polypeptide in these particles, but it was definitely not 517 amino acids in length (Figure 6.4.1). It can be seen that the truncated protein band from cells carrying pGTR517 is running at a higher molecular mass on the gel than the GroEL531 band. This suggests that some sort of change had occurred to the

groEL517 gene during the cloning, possibly as the result of a small insertion or duplication. It should be remembered that pGTR517 was isolated from a lone colony after transformation with M13ESL8–517/pJF118 ligation mix. It could be that even in a *pcnB* strain the GroEL517 gene product causes sufficiently severe biological problems so that only a mutant form of the protein was recovered.

Figure 6.4.1 SDS–PAGE of GroEL particles purified from cells carrying pGTR548, pGTR531 and pGTR517.



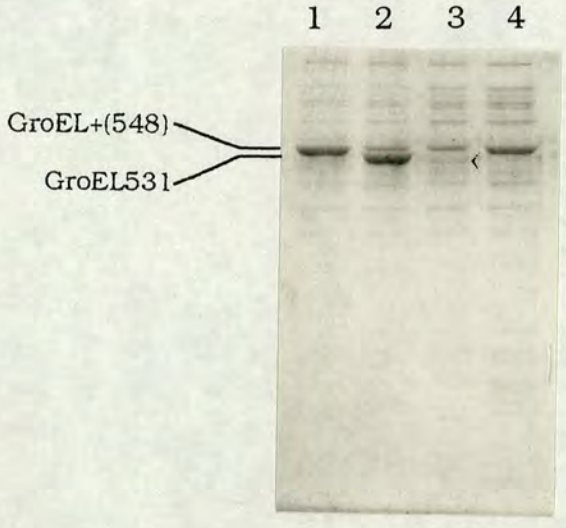
M	molecular weight markers
1	GroEL particles from MM38pcn (pGTR548)
2	GroEL particles from MM38pcn (pGTR531)
3	GroEL particles from MM38pcn (pGTR517)

GroEL particles were isolated from the given strains and subjected to SDS–PAGE as described in the *Materials and Methods*. Cells carrying pGTR548 show a single GroEL band. Cells carrying pGTR531 and pGTR517 show mixed GroEL particles, but unexpectedly the peptide produced from pGTR517 is apparently larger than that from pGTR531.

6.5 Purification of GroEL particles from cells infected with M13ESL8-derivatives

There seemed to be no problem in expressing correct length GroEL517 in cells carrying M13ESL8–517. Since this was the case it was decided to purify the GroEL tetradecamers from cells infected with the M13ESL8 derivatives. The GroEL particles were purified as before and subjected to SDS–PAGE. Figure 6.5.1 shows that cells infected with M13ESL8–548 produce wild-type GroEL particles, and M13ESL8–531 produces particles containing both the wild-type and truncated polypeptides.

Figure 6.5.1 SDS–PAGE of GroEL particles purified from TG1 infected with M13ESL8 derivatives.

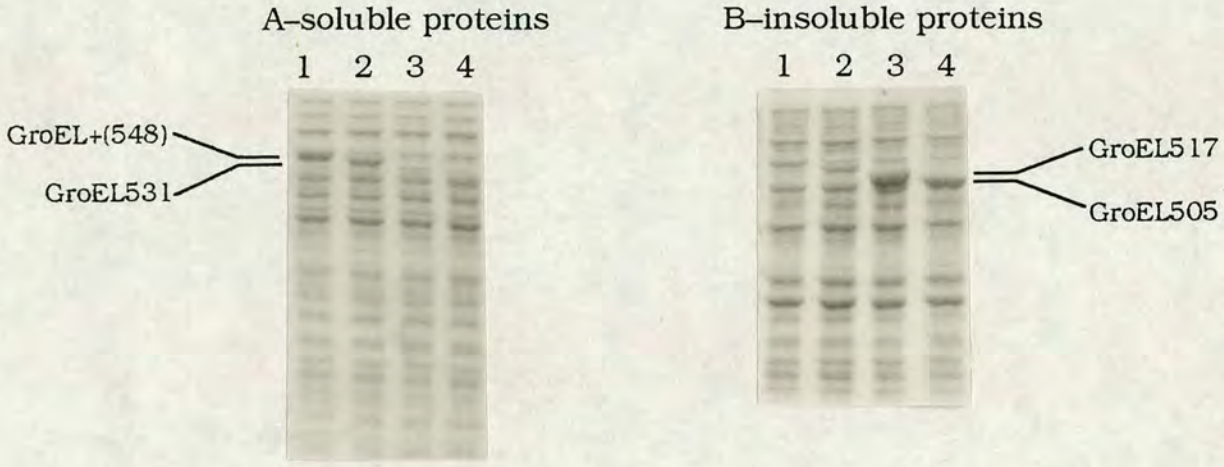


1 GroEL particles from TG1 infected with M13ESL8–548
2 GroEL particles from TG1 infected with M13ESL8–531
3 GroEL particles from TG1 infected with M13ESL8–517
4 GroEL particles from TG1 infected with M13ESL8–505

GroEL particles were purified from TG1 cultures infected with the given M13 lysate. M13ESL8–548 produces a single GroEL species (lane 1). The sample from M13ESL8–531 demonstrates heterogenic GroEL particles (lane 2). The particles isolated from M13ESL8–517 are mainly composed of full-length GroEL, but a small amount of GroEL517 is found in the preparation (lane 3). No GroEL505 was found in particles isolated from cells infected with M13ESL8–505 (lane 4). This was verified by Western blotting (data not shown).

However, for M13ESL8–517-infected cells only a tiny amount of truncated GroEL protein could be detected in the GroEL particle fractions. For M13ESL8–505 no truncated GroEL could be found in GroEL preparations, thereby corroborating the results obtained with pGTR505. Further analysis revealed that the GroEL517 and 505 proteins were to be found in the insoluble fraction of the cell extract (Figure 6.5.2).

Figure 6.5.2 SDS–PAGE of soluble and insoluble protein fractions from TG1 infected with M13ESL8 derivatives.



1	TG1 infected with M13ESL8–548
2	TG1 infected with M13ESL8–531
3	TG1 infected with M13ESL8–517
4	TG1 infected with M13ESL8–505

Cultures of TG1 were infected with the given M13 phage and incubated for six hours at 37°C. The soluble and insoluble protein fractions were isolated and subjected to SDS–PAGE as described in the *Materials and Methods*. Note that GroEL548 (lane 1) and GroEL531 (lane 2) proteins are found in both soluble and insoluble fractions, whereas GroEL517 (lane 3) and GroEL505 (lane 4) are found primarily amongst the insoluble proteins.

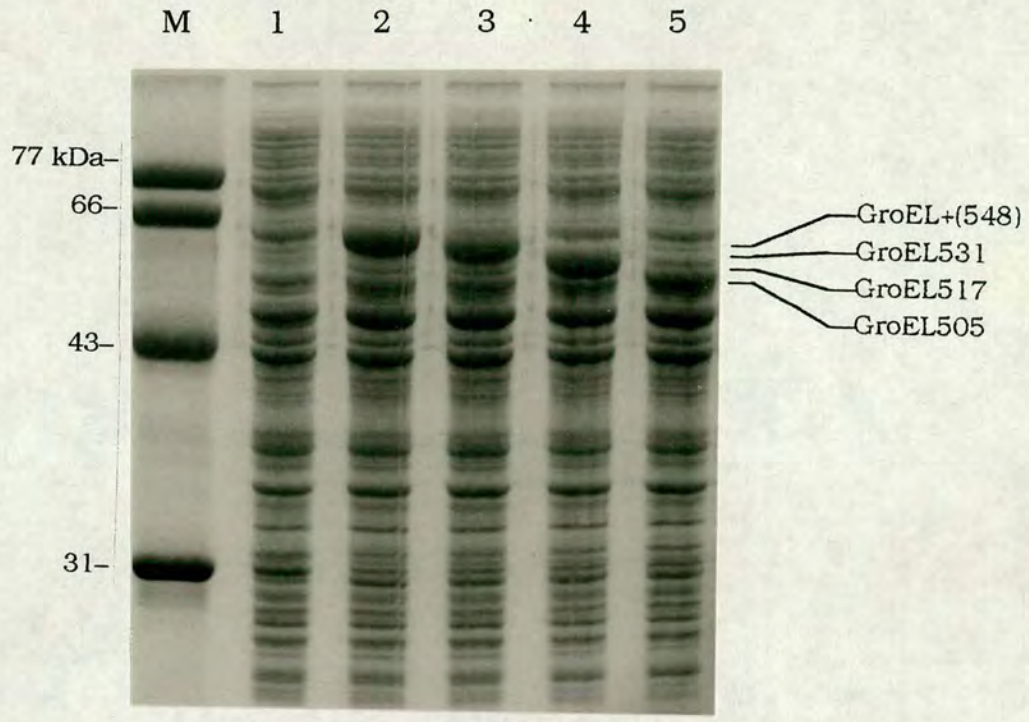
This suggested that these polypeptides were stable but had precipitated or aggregated, possibly because of misfolding in the cell but it is possible that some of the GroEL517 protein is incorporated into the GroEL tetradecamers although most of this material is insoluble. Strangely cells infected with M13ESL8–505 seemed to

contain more wild-type GroEL than cells infected with M13ESL8–531 or 517 (although this may be artefactual since the purification was only performed once). It has previously been shown that production of aberrant polypeptides in *E. coli* can induce a heat-shock response (Goff and Goldberg, 1985) and it is possible that the production of GroEL505 results in a heat-shock response and that GroEL is thus induced. GroEL517 did not produce the same effect.

6.6 Cloning the M13ESL8-derivative DNA inserts into a mini-F plasmid

My last efforts to obtain information about the serial truncations of GroEL involved cloning the SDM-derived DNA inserts from M13ESL8 into a very low-copy-number plasmid. A mini-F plasmid was chosen as the recipient for the inserts. F plasmids should have a copy number of one molecule per cell and it was hoped that this would produce the truncated forms of GroEL at a low gene dosage, which would be less harmful for the cell, and might yield information unobtainable using M13-based systems. Plasmid pHR277, kindly given by Hiroshi Hara, is a mini-F plasmid with unique *Hin*dIII and *Eco*RI restriction sites. The 2.1 kb *groE*-containing *Hin*dIII–*Eco*RI fragments from M13ESL8–548, 531, 517 and 505 were gel purified and ligated to pHR277 that had been restricted with the same enzymes. Ligation mixes were transformed into DL307 since this strain often gave very high transformation efficiencies. All the transformations resulted in 20–50 colonies. Plasmid DNA was extracted from a selection of these, restricted with *Hin*dIII and *Eco*RI and analysed by agarose gel electrophoresis. Owing to the low copy number of mini-F plasmids the DNA gels were hard to interpret, but for the most promising of the transformants whole-cell extracts were analysed by SDS–PAGE. In this way I was able to visualize the different forms of the GroEL proteins present and also confirm that each mutant was producing proteins of the correct length (Figure 6.6.1). The *groE*-containing mini-F plasmids made were called pLT548, 531, 517 and 505.

Figure 6.6.1 SDS–PAGE of total protein from cell extracts carrying pLT plasmids.



M	molecular weight markers
1	DL307(pHR277) whole-cell extract
2	DL307(pLT548) whole-cell extract
3	DL307(pLT531) whole-cell extract
4	DL307(pLT517) whole-cell extract
5	DL307(pLT505) whole-cell extract

Whole-cell extracts were isolated from the given strains and subjected to SDS–PAGE as described in the Materials and Methods. The decreasing sizes of the GroEL proteins produced from the plasmids can clearly be seen.

Genetic analysis of the pLT plasmid series

Plasmid pHR277 and the pLT plasmid series were each transformed into NL441 (*groEL44*^{ts}), NL13 (*groE*$^{+}$), and NM306 (*groE*$^{+}$, *purA*). The NL441 transformed cells were used to examine any complementing activity caused by the mini-F-encoded *groEL'* genes. It was found that only pLT548 and pLT531 complemented the *groEL44* mutation both for high-temperature growth and phage plating (Table 6.6.1).

Table 6.6.1 Complementation of NL44 by pLT plasmids.

	Colony size		λ plaque size	
Strain	**30°C**	**42**	**30°C**	**42**
NL44(*groEL44*)				
pHR277	+++	-	-	na
pLT548	+++	+++	+++	+++
pLT531	+++	+++	+++	+++
pLT517	+++	-	-	na
pLT505	+++	-	-	na

Strains were streaked out on L-agar and incubated at the given temperatures overnight and examined for growth. Dilutions of phage λ were spotted onto BBL top-agar lawns of the given strains, incubated overnight and examined for plaque formation. +++, well formed colonies/plaques. -, no colonies/plaques visible.
na. Not applicable.

A similar result was obtained when the pLT plasmids were used in an attempt to complement a *groE* chromosomal deletion. NM306 carrying each of the plasmids was transduced from *purA* to *purA*$^+$ using a P1 lysate grown on NL192Ω (in which the *groE* locus is replaced by the spectinomycin/streptomycin-resistance-encoding Omega (Ω) fragment (see *Chapter 4*)). All the strains could be transduced to *purA*$^+$ but only those carrying pLT548 and pLT531 demonstrated coinheritance of the Ω fragment (Table 6.6.2). From these results it can be concluded that to obtain genetic complementation the GroEL protein expressed must be of a still undefined length somewhere between 517 and 531 amino acids.

Table 6.6.2 Transduction of NM306 with P1 NL192Ω(*groEΔ*::Ω).

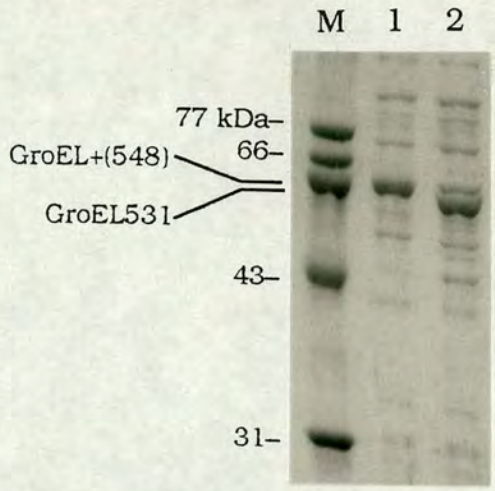
Recipient strain	No. of *purA*$^+$ progeny	No. of Str^R/Spc^R progeny	Linkage (%)
NM306 pHR277	120	0	0
pLT548	130	21	16
pLT531	146	22	15
pLT517	110	0	0
pLT505	122	0	0

NM306 carrying the given plasmids was transduced with P1 {NL192Ω(*groEΔ*::Ω)} and selection made for *purA*$^+$ transductants using VB-supplemented minimal agar lacking adenine. All the resulting *purA*$^+$ clones were patched on agar containing streptomycin/spectinomycin at 30°C, incubated overnight and scored for growth. Only those cells which contain functional GroE proteins will inherit Str^R/Spc^R.

Purification of GroEL particles from cells carrying the pLT plasmids

Since my previous work had suggested that GroEL517 (and to some extent 505) might be interfering with normal GroEL function *in vivo* it was decided to purify GroEL particles from cells carrying the pLT plasmid series. The viability of cells carrying these low-copy-number plasmids seemed normal and, unlike the other systems, no problems had been encountered with strains carrying either pLT517 or pLT505. GroEL particles were purified as before from both NL13 and DL307 carrying pLT548, 531, 517 and 505 and subjected to SDS–PAGE and Western blot analysis. For both strains cells carrying pLT548 and 531 gave the expected banding patterns in that pLT548 produced a single GroEL band and pLT531 produced a doublet (Figure 6.6.2).

Figure 6.6.2 SDS–PAGE analysis of purified GroEL particles from cells carrying pLT548 and pLT531.

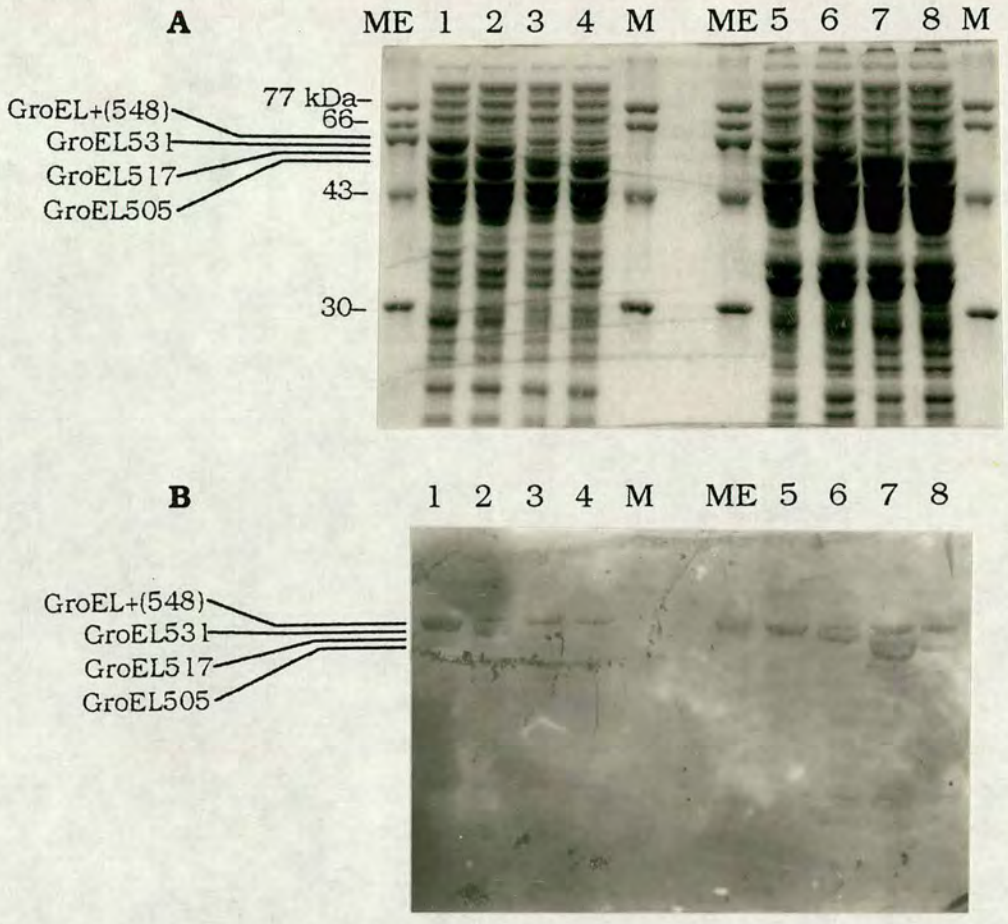


M	molecular weight markers
1	DL307(pLT548)–Purified GroEL
2	DL307(pLT531)–Purified GroEL

GroEL proteins were purified from DL307 and subjected to SDS–PAGE as described in the Materials and Methods. Particles from cells carrying pLT548 show a single wild-type GroEL band (lane 1). Particles from cells carrying pLT531 show a double GroEL band with the major band corresponding to the truncated form of the protein.

In strains carrying pLT505 the presence of the truncated GroEL505 in the GroEL-tetradecamer-containing fractions could not be demonstrated and this polypeptide, as before, was only found in the insoluble fraction of the cell extract (Figure 6.6.3). It was also noted that GroEL505 was not present in equal amounts to any of the other forms of GroEL when produced from these plasmids. It could be that GroEL505 is unstable and a substrate for proteases. It has been stated previously that GroEL505 production may induce a heat-shock response. This may facilitate its degradation.

Figure 6.6.3 SDS–PAGE and Western blot analysis of soluble/insoluble proteins from cells carrying pLT plasmids.

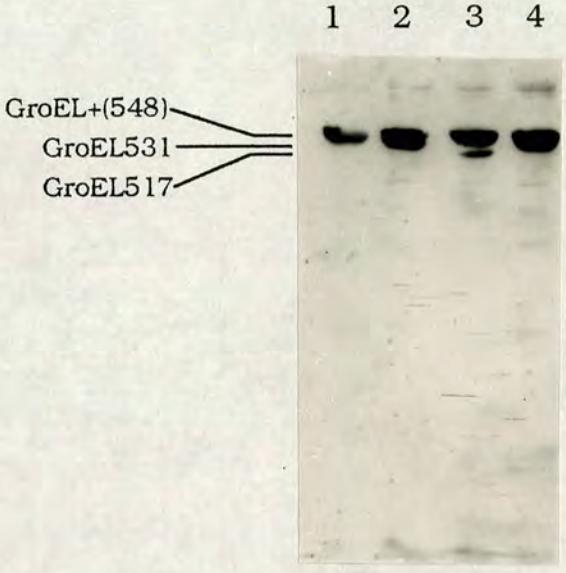


M	molecular weight markers
ME	molecular weight markers + GroEL
1/5	DL307(pLT548)
2/6	DL307(pLT531)
3/7	DL307(pLT517)
4/8	DL307(pLT505)

Cultures of DL307 with the given plasmids were incubated to late log-phase at 37°C. The soluble (lanes 1–4) and insoluble (lanes 5–8) protein fractions were isolated and subjected to (A) SDS–PAGE and (B) Western blot analysis as described in the *Materials and Methods*. Note that most of the GroEL548 (lane 1/5) and GroEL531 (lane 2/6) proteins are found distributed between the soluble and insoluble cell fractions, whereas GroEL517 (lane 3/7) and GroEL505 (lane 4/8) are only found amongst the insoluble proteins. Note that the amount of GroEL505 found in lane 8, compared to the amounts of the other GroEL' proteins present, suggest that it is underproduced or degraded.

With cells carrying pLT517 differences were found between NL13 and DL307. In DL307 the GroEL517 protein was never recovered from GroEL particles (after several attempts with cells grown at 30 and 37°C) and was only recovered from the insoluble material. However, with NL13(pLT517) although a certain amount of GroEL517 was found in the insoluble fraction (as is GroEL wild-type) some was also detected copurifying with the GroEL tetradecamers (Figure 6.6.4).

Figure 6.6.4 Western blot analysis of purified GroEL particles from NL13 carrying pLT plasmids.



1 NL13(pLT548) purified GroEL particles
2 NL13(pLT531) purified GroEL particles
3 NL13(pLT517) purified GroEL particles
4 NL13(pLT505) purified GroEL particles

Cultures of NL13 carrying the given plasmids were incubated at 37°C to late log-phase. GroEL particles were isolated, subjected to SDS–PAGE, electroblotted onto nitrocellulose and probed with anti-GroEL antibodies as described in the *Materials and Methods*. NL13(pLT548) produces a single GroEL species (lane 1). The sample from NL13(pLT531) demonstrates a heterogenic GroEL preparation (although this is not too clear from the reproduction, lane 2). The particles isolated from NL13(pLT517) are mainly composed of full-length GroEL, but a small amount of GroEL517 is found in the preparation (lane 3). No GroEL505 was found in particles isolated from NL13(pLT505) (lane 4).

This suggested that the GroEL517 polypeptide contained enough information to be incorporated into GroEL tetradecamers (whereas GroEL505 does not) even though it is likely to impair their functionality.

6.7 Summary and discussion

Previous experiments using a variant of GroEL which had the carboxyl-terminal 18 amino acid GM tail removed had demonstrated that this tail seemed to be virtually dispensable for normal function, and only under certain conditions were cells compromised by its absence. To conclude this work it was decided to further shorten the GroEL protein in order to discover how much of the protein would have to be removed before loss of function was observed. The GroEL sequence encoded by M13ESL8 was manipulated using site-directed mutagenesis, and in-frame TAA STOP codons were incorporated into the protein resulting in GroEL molecules of 505, 517, and 531 amino acids (the intact protein comprises 548 amino acids).

Since the M13ESL8 mutagenized phages were producing the GroEL proteins of predicted lengths, it was decided to purify GroEL tetradecamers from cells infected with these phages to see whether the truncated GroEL subunits are assembled into tetradecamers. This, and cell-extract analysis suggested that most of the GroEL517 and 505 protein produced from the M13ESL8 clones was insoluble, but very small amounts of soluble GroEL517 were seen in the GroEL preparations suggesting that the protein may indeed contain enough information to be assembled.

The inserts from the M13ESL8 derivatives were cloned into the very low-copy-number F'-based plasmid pHR277 resulting in the pLT plasmid series which, unlike M13-based clones, are suitable for genetic complementation tests. It was found that the *groEL* genes on pLT548 and pLT531 could complement both *groEL*^{ts} and *groEL*-deletion mutants for high-temperature growth and phage morphogenesis, but those from pLT517 and pLT505 could not. It can be said therefore that the cut-off point for a functional GroEL protein is somewhere between 518 and 531 amino acids. This finding is consistent with work by A. Horwich which suggests that the minimum

length of a functional GroEL protein *in vivo* is 520 amino acids (cited in Langer *et al.*, 1992b).

Purification of GroEL particles from cells carrying the pLT plasmids showed that GroEL548 and GroEL531 were assembled into GroEL tetradecameric particles. In the case of GroEL531 it seemed likely that the GroEL particles were heteromeric, comprising plasmid-encoded GroEL531 and chromosomally encoded GroEL548 polypeptides. GroEL505 was never found in GroEL particles and was only recovered from insoluble cellular material. Plasmid pLT517, however, gave conflicting results. In all cases much of the GroEL517 was found in insoluble protein fractions. Analysis of purified GroEL particles from strains carrying pLT517 showed that in one strain (NL13) the GroEL517 polypeptides are found in GroEL preparations, and in another strain (DL307) they are not and the subunits are found to be insoluble. However, the fact that GroEL517 can be recovered from GroEL tetradecamers under any circumstances shows that it is assembly competent. The differences seen between DL307 and NL13 are possibly caused by NL13 having a slower growth rate than DL307, which is a rapidly growing strain. It should be possible to find the ideal growth conditions so as to maximize GroEL517 incorporation into GroEL tetradecamers. Such particles might prove interesting if subjected to electron microscopy and peptide-folding assays.

One potential problem with this work came to light after a publication which showed that apparently pure GroEL preparations could contain large amounts of non-GroEL protein (Hayer-Hartl and Hartl, 1993). It is suggested that owing to its nature as a peptide-binding protein, other minor proteins will be present in GroEL preparations. In my case GroEL tetradecamers were isolated from cells which were not overexpressing the protein and so contaminating proteins were found in the GroEL-containing fractions. Perhaps GroEL517 is not actually assembled into GroEL teradecamers but is only associated with the tetradecamers as an unfolded polypeptide. Attempts were made to separate the GroEL548 tetradecamers from GroEL517 polypeptides by incubating the preparations in the presence of Mg–ATP, but no effects were seen on the preparations (data not shown). If GroEL517 were only a contaminating band in

GroEL preparations then one might expect to find the GroEL505 polypeptides in GroEL tetradecamers isolated from cells carrying pLT505, but this is not seen. The GroEL517 protein is sometimes found as a major band in the GroEL preparations in which it occurs, in comparison with levels of non-GroEL proteins that are present.

Examination of the sequences removed in this analysis (Figure 6.6.1) shows that of the 14 amino acids lost between GroEL517 and GroEL531, six are highly conserved. This region of the protein must be important for normal GroEL function *in vivo*. As stated above Horwich finds the minimum length for GroEL function is 520 amino acids. The only highly conserved amino acid found between 517 and 520 is glutamic acid–518. Perhaps this residue is absolutely required for GroEL function *in vivo*. (I am unaware whether Horwich made GroEL518 or GroEL519.) Of the 12 amino acids lost between GroEL505 and GroEL517 ten are highly conserved. This high degree of conservation suggests an important role which, as indicated from the results here, might be concerned with assembly of the GroEL tetradecamer. Langer *et al.* (1992b) have shown that treatment of wild-type GroEL tetradecamers (stabilized with either ATP or ADP) with proteinase K *in vitro* results in the release of a ~50 amino acid long fragment from each of the 14 GroEL carboxyl-termini (giving GroEL<500). These particles are apparently homogeneous consisting only of truncated GroEL, are stable, bind GroES and can promote the folding of rhodanese. They do, however, have a reduced ATPase activity compared to wild-type particles. This suggests that GroEL505 is potentially active, but lacks the ability to be assembled into GroEL tetradecamers possibly owing to the loss of some key assembly sequence. Alternatively, this sequence may be involved in folding of the GroEL monomers, and its removal causes misfolding. Thus the lack of assembly may be a consequence of this. GroEL517 contains this sequence but is still non-functional with respect to some vital reaction requiring GroEL function. Indeed, too much GroEL517 seems to be toxic to the cell presumably because it is interfering in normal GroEL interactions.

Attempts to clone DNA encoding the GroEL517 protein in high-copy-number plasmids failed. When the plasmid copy number was reduced by introduction of the *pcnB*::*kan* gene a single clone was

isolated. Further analysis of this clone demonstrated that a mutation had occurred resulting in a GroEL' species of between 530–545 amino acids. This clone was not analysed further, but was of interest since it was biologically toxic, and seemed to assemble into GroEL tetradecamers efficiently. Future characterization of this mutant might prove intriguing.

Figure 6.6.1 Amino acids 490–536 from the carboxyl-terminal region of GroEL showing the highly conserved amino acids.

....Asp Met Gly **Ile** Leu Asp **Pro** Thr **Lys Val** Thr **Arg** Ser **Ala Leu Gln**^{505} Tyr **Ala Ala Ser Val Ala**

Gly Leu Met **Ile Thr Thr**^{517} **Glu** Cys Met Val **Thr** Asp **Leu Pro Lys** Asn Asp Ala **Ala** Asp^{531} Leu

Gly Ala **Ala Gly**....

Amino acids 490–536 from *E. coli* GroEL. The amino acids indicated in bold are highly conserved among several of the HSP60. This was determined using the PILE-UP application from the Wisconsin GCG computer software (Genetics Computer Group, 1991) on 15 randomly chosen HSP60 DNA sequences to generate a consensus sequence that was translated and compared to GroEL.

CHAPTER 7
CONCLUSIONS AND PERSPECTIVES

The initial intention of this project was to continue the work of Jenkins (1985) and March (1988) that concerned the phenomenon of suppression of *dnaA*^{ts} alleles by the overexpression of the *groE* genes. However, as so often happens with open-ended research, the subject matter wandered, following the most interesting observations, and consequently this thesis mainly concerns characterization of a specific *groEL* mutant.

Chapter 3 describes the work that followed on directly from the research of March (1988). This involved 'improvement' of a truncated form of *groEL* which March described as being more efficient than wild-type at suppressing *dnaA*. I constructed a clone which expressed a truncated form of GroEL lacking the 16 amino acid carboxyl-terminal glycine–glycine–methionine motif. It was the removal of this sequence which March believed allowed his truncated form of *groEL* to suppress *dnaA* better than wild-type *groEL*. Much to our surprise, my *groEL*$_{\mathbf{tr}}$ clone did not suppress *dnaA* mutations, but did allow complementation of *groEL*^{ts} mutations. This was very unexpected considering the highly conserved nature of this motif within the GroEL-like protein family. One would have thought that highly conserved motifs would be critical for protein function. There are cases where overexpression of functionally impaired proteins can suppress temperature-sensitive mutants, but *groEL*$_{\mathbf{tr}}$ can complement *groEL*^{ts} mutations when present in the cell at low copy. In this chapter I also show that the original conclusions made by March from his observations were probably wrong, however, they did lead to my discovery of the apparent dispensability of an unusual and highly conserved region of the GroEL protein. It was this observation that was to be the crux of this project, rather than the observation concerning the lack of suppression of *dnaA* by *groEL*$_{\mathbf{tr}}$. However, this suppression/lack of suppression is still interesting. Future work (inspired by Horwich *et al.*, 1993) could examine the solubility of the DnaA46 protein in suppressed and unsuppressed systems.

Chapter 4 mainly concerns the construction of a *groE*-deletion mutant and its use in complementation experiments involving *groEL*$_{\mathbf{tr}}$. Since the GroEL protein's functional form is a tetradecameric

particle, it was felt that the complementation of *groEL*^{ts} mutants by *groEL*$_{tr}$ could be caused by the formation of mixed multimers forming between the temperature-sensitive and truncated polypeptides, resulting in functional, temperature-resistant GroEL particles. We wanted to know whether *groEL*$_{tr}$ could complement a *groE* deletion strain. After some difficulties a *groE* deletion strain was constructed and verified by DNA/protein analysis. It was found that *groEL*$_{tr}$ could complement this strain, demonstrating that the GM tail of GroEL was dispensable from the cell, at least for growth under the conditions used for its isolation.

Chapter 5 was a search for a phenotypic difference between strains expressing either wild-type or truncated forms of *groEL*. Several parameters were examined in which it was believed that the GroE proteins might be involved. At first no differences could be found between the strains in their growth, utilization of diverse carbon sources, phage plating capacity, UV resistance and β-lactamase excretion. The combination of *dnaA46* and *groEL*$_{tr}$ was found to be slightly poorer growing than *dnaA46* and *groEL*$^{+}$ but the differences were marginal; however this could be worthy of further investigation. The first evidence of a difference between the strains was from the competition experiments which showed that cells expressing the wild-type form of *groEL* were at an advantage, especially when incubated at 42°C. This advantage was found to be caused by the strain expressing *groEL*$^{+}$ coming out of stationary phase more rapidly than those expressing *groEL*$_{tr}$. Future work could attempt to address in what way these populations of cells differ (e.g. 2-D PAGE analysis of protein-profiles of stationary-phase cultures of these strains). Collaborative research showed that the only biochemical difference found between GroEL$_{tr}$ and GroEL^{+} *in vitro* was that the truncated form of the protein hydrolysed ATP 1.5 times more slowly than wild-type. I have discussed how this difference in the rates of ATP hydrolysis could explain our observations. Nevertheless, this still leaves unanswered questions, especially those concerned with the conservation of the GM motif. Luck led to the discovery of the sensitivity of *groEL*$_{tr}$ expressing strains towards the dye crystal violet (and certain other antibacterial agents). This not only suggests that the GroEL proteins are involved

with some aspect of membrane biogenesis, but also suggests a requirement for the GM tail in this function. I feel that this reflects the cells requirement for a GM-containing GroEL protein in real-life situations, when the cell is constantly challenged by potentially harmful environmental agents, and hence its dispensability in the pampered environment of the laboratory. Re-examination of 1-D SDS–PAGE gels of membrane samples, from cells carrying either GroEL$^+$ or GroEL$_{tr}$ which I had run earlier (to see whether GroEL was membrane-associated) showed no obvious differences. However, the small amount of GroEL present in the samples did seem to consist of two forms. In light of the crystal violet effect it would be very interesting to examine this phenomenon more fully. Examination of the membrane proteins using 2-D PAGE might also prove enlightening.

Chapter 6 dealt with the further truncation of the *groEL* gene with an aim of discovering how much more of the carboxyl-terminus was dispensable before a loss of function was observed. Site-directed mutagenesis was used to construct *groEL* genes which would produce proteins of length 531 (which was almost identical to the original truncation made and thus acted as an internal control), 517 and 505 amino acids. GroEL531 behaved as expected, supporting growth and assembling into GroEL particles. Neither GroEL517 nor GroEL505 could complement temperature-sensitive or deletion mutants, and both were problematic to clone in high copy. GroEL505 was completely insoluble, and was never seen associated with GroEL tetradecamers. GroEL517 tended to be insoluble, but a fraction of it was found to be associated with GroEL particles. I have proposed that there could be some sequence removed from GroEL505, which is present in GroEL517, that is required for GroEL assembly, and the GroEL505 aggregates because it is not assembled. However, it is just as possible that GroEL505's propensity to aggregate causes its lack of assembly. The GroEL517 protein may be of future interest. The soluble yields of this subunit could potentially be improved upon by maximizing growth conditions, and cloning in high-copy number (e.g. *groEL517* may be maintained at high copy in the cell when *groE*$^+$ is also present at high copy).

Amongst many other things, this project has taught me that molecular biology is an unpredictable science, always posing new problems and abiding to its own set of rules. This used to frustrate me, but I (slowly) accepted this and realised that without unpredictability, science would be boring and experimental research would become redundant. There is something strangely wonderful about the feeling of bewilderment one experiences when an unexpected effect is seen, especially when a strain of *E. coli* decides to grow or not. I sometimes try to visualize the insides of the bacterial cell, which makes me wonder how much can be learned from the piece-by-piece dissection of such a complex system that we are far from understanding. The biochemical research into GroEL has so far outpaced the genetic studies, however, biochemists usually know the exact contents of their experimental test-tubes, whereas molecular biologists working with *in vivo* systems do not. To quote an almost famous author, "It's like monkeys dismantling a TV set to find out where the picture comes from!" Even so, I am at-a-loss as to suggest a better approach as yet, but I am certainly not suggesting that less attention should be paid to *in vivo* research; there should be more, lots more. I hope that readers who have managed to reach this far have found my research as interesting and stimulating (but not as frustrating) as I have.

Chapter 8
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APPENDIX

Published Work

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The strongly conserved carboxyl-terminus glycine–methionine motif of the *Escherichia coli* GroEL chaperonin is dispensable

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Summary

The universally distributed heat-shock proteins (HSPs) are divided into classes based on molecular weight and sequence conservation. The members of at least two of these classes, the HSP60s and the HSP70s, have chaperone activity. Most HSP60s and many HSP70s feature a striking motif at or near the carboxyl terminus which consists of a string of repeated glycine and methionine residues. We have altered the *groEL* gene (encoding the essential *Escherichia coli* HSP60 chaperonin) so that the protein produced lacks its 16 final (including nine *gly*, and five *met*) residues. This truncated product behaves like the intact protein in several *in vitro* tests, the only discernible difference between the two proteins being in the rate at which ATP is hydrolysed. $GroEL_{tr}$ can substitute for GroEL *in vivo* although cells dependent for survival on the truncated protein survive slightly less well during the stationary phase of growth. Elevated levels of the wild-type protein can suppress a number of temperature-sensitive mutations; the truncated protein lacks this ability.

Introduction

The GroEL protein is the *Escherichia coli* member of the universally conserved HSP60 (heat-shock protein) family of chaperone proteins (for recent reviews see Ellis and van der Vies, 1991; Zeilstra-Ryalls *et al.*, 1991). These essential proteins are thought to mediate the correct folding of polypeptides, probably by binding to nascent or unfolded molecules and so preventing undesirable amino acid interactions within or between them (Laminet *et al.*, 1990). Although GroEL is known to adopt a 14-mer structure in which two seven-membered rings are thought to surround a central space (Saibil *et al.*, 1991), the actual protein–protein interactions which are responsible for this structure, or for the binding of substrate protein molecules, are unknown. The amino-acid sequence of GroEL (Hemmingsen *et al.*, 1988) is unremarkable except that GroEL and almost all other members of the HSP60 family (other than the chloroplast Cpn60s) share a striking motif located at or near the carboxy-terminus of the protein (Fig. 1). This motif, variable in length and exact sequence, consists mainly of glycine and methionine residues. Many members of the HSP70 family also share a similar motif (Fig. 1).

Because strongly conserved protein motifs are thought likely to have functional significance, and because the mechanisms of action of HSPs are of considerable current interest, we decided to investigate the function of this *C*-terminal protein motif by engineering a truncated form of GroEL ($GroEL_{tr}$) which lacks this tail sequence. Our results show that although the tail is apparently dispensable during exponential growth, cells which produce the native form of GroEL out-compete cells which must rely on the truncated form.

Results

Constructing a truncated form of GroEL

The GroEL protein of *E. coli* is 548 amino acids long and ends with the sequence GGMGGMGGMGGMM (Hemmingsen *et al.*, 1988). Preliminary experiments had shown that a fusion protein, which consists of the first 496 amino acids of GroEL and a terminal region composed of 86 amino acids derived from the out-of-frame sequence of the *tet* gene of pBR325, is not functional. The construct which expresses this fusion protein, pJM32 (March, 1988), could not complement *groEL(ts)* mutants, suggesting that a normal carboxy-terminal region may be essential for protein function.

Encouraged that this glycine and methionine-rich *C*-terminal motif (hereafter referred to as the GMR motif)

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HSP 60s

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                          ----------------
E. coli.      TDLPKNDAADLGAAGGMGGMGGMGGMM   548
                          --------------
Yeast.        VAIVDAPEPPAAAGAGGMPGGMPGMPG   570
                          ----------------
Rat.          TEIPKEEKDPGMGAMGGMGGGMGGGMF   547
                          ----------------
Human.        TEIPKEEKDPGMGAMGGMGGGMGGGMF   573
                          ---------
My. tub.      TEAVVADKPEKEKASVPGGGDMGGMGF   540
                          ----------------
Ps. aerug.    VAEIVEDKPAMGGMPDMGGMGGMGGMM   547
                          ------------------
Cox. bur.     PKKKEESMPGGGDMGGMGGMGGMGGMM   552
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HSP 70s

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                ------------------------------------------
LD mildew GKMYAAAGAAGGMPGGMPGMPGGMPGMPGGMPGMPGGMPGGMPGGMPGGWPPCWSSDQGPKIEEVD 675
                ----------------------------------
T. brucei LSKMYQGMGGGDGPGGMPEGMPGGMPGGMPGGMGGGMGGAAASSGPKVEEVD              661
                ----------------------------------------
P. falc.  GDAAGAAGGMPGGMPGGMPGGMPSGMPGGMNFPGGMPGAGMPGNAPAGSGPTVEEVD          681
                ---------------------------------
Fruit fly EGVCNPIITKLYQGAGFPPGGMPGGGGGMPGAAGAAGAAGAGGAGPTIEEVD              651
                ------------------------
Human     EKVCNPIITKLYQSAGGMPGGMPGGFPGGGAPPSGGASSGPTIEEVD                   646
```

Fig. 1. The carboxyl-terminal sequences of representative HSPs with GMR carboxyl-terminal repeats. The PIR protein data-base, Version 23 (George *et al.*, 1986), was searched for matches to the sequence GGMGGMGGMGG using the methods described in Smith and Waterman (1981) and Lyall *et al.* (1985). The sequences listed above (with the exception of the bacterial HSP60s whose sequences were not included in the data-base) were amongst those found. Their data-base accession numbers are, from top to bottom, HSP60s: S01432, HHRT60, A32800, JQ0157. HSP70s: A25398, HHUM7B (Lettuce downy mildew), JU0164, A36333, A27077. The sequences for other HSP60s are *Mycobacterium tuberculosis*, Shinnick (1987), *Pseudomonas aeruginosa*, Sipos *et al.* (1991), and *Coxiella burnettii*, Vodkin and Williams (1988).

might be essential for GroEL function, we engineered the *groEL* gene so that a truncated protein would be produced. This was done by cutting at a *Pvu*II site located near the 3' end of the *groEL* gene (see Fig. 6 later) in pIR88 and inserting a linker with termination codons in each of the three possible reading frames. A truncated protein ($GroEL_{tr}$) was produced from this construct, pHC18, and is clearly distinguishable as smaller than the wild-type protein ($GroEL_{wt}$) on SDS–PAGE gels (Fig. 2). Note that the amounts of pHC18-encoded truncated protein and pIR88-encoded normal protein produced are comparable, indicating that the truncated protein is not significantly degraded *in vivo.*

groEL$_{tr}$ was cloned in both high- and low-copy-number vectors (Table 1). Strains containing these plasmids grow normally at 30°C and 42°C and are able to plate λ and T4 (GroEL mutants cannot support growth of these phages, (Georgopoulos *et al.*, 1973)). Thus $GroEL_{tr}$ does not appear to interfere with the normal activity of $GroEL_{wt}$, even when produced at high levels.

Isolation and in vitro *properties of the truncated protein*

NL13(pHC18) was used to prepare $GroEL_{tr}$ protein in the native 20S oligomeric form (Bochkareva *et al.*, 1992). The S-values for $GroEL_{wt}$ and $GroEL_{tr}$ cannot be distinguished. Figure 3 shows an SDS–PAGE gel on which the NL13(pHC18) 20S extract, an extract similarly prepared from strain HB101(pGroE4) and containing $GroEL_{wt}$, and a mixture of the two have been electrophoresed. The NL13(pHC18) preparation consists entirely, in so far as can be measured, of a protein which is smaller than, and

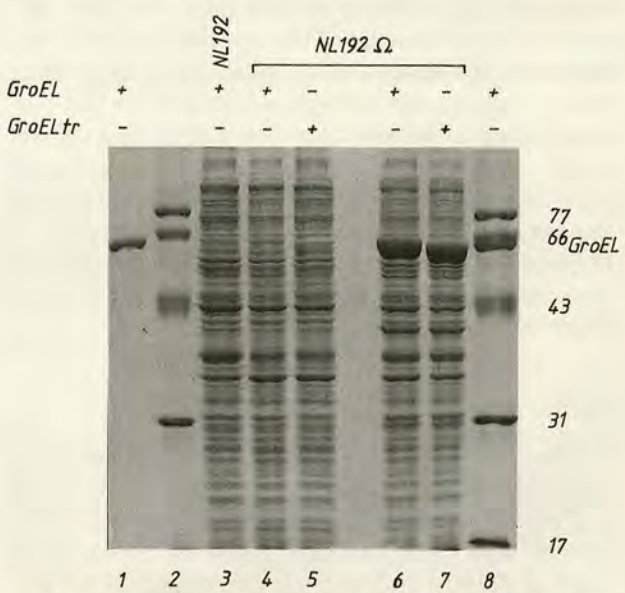

Fig. 2. Coomasie brilliant blue-stained 10% SDS–PAGE of strains producing $GroEL_{wt}$ or $GroEL_{tr}$. Lanes: 1, purified $GroEL_{wt}$; 2, 8, molecular mass markers, sizes in kDa; 3, control strain with pML31; 4–7, *groELS* chromosomal deletion strain with plasmids; 4, pIRF1; 5, pHCF3; 6, pIR88; 7, pHC18.

Fig. 3. 10% SDS–PAGE of 20S GroEL particles. Lanes: 1, $GroEL_{wt}$ from HB101(pGroE4); 2, $GroEL_{tr}$ from NL13 (pHC18); 3, mixture of the above. A very faint band of $GroEL_{wt}$ could be seen in lane 2 on the original gel.

clearly distinguishable by size from, the wild-type protein, demonstrating that $GroEL_{tr}$ can form normally sized oligomers. The fact that this preparation contains almost no detectable $GroEL_{wt}$ suggests that the copy number of pHC18 is high.

The $GroEL_{tr}$ preparation was compared with a $GroEL_{wt}$ preparation to show that absence of the *C*-terminal tail has no destabilizing effect on the GroEL oligomer *in vitro*. This was shown in two ways (Lissin *et al.*, 1990). First, the resistances of $GroEL_{tr}$ and $GroEL_{wt}$ to monomerization by 3 M urea at 0°C are very similar; conversion of oligomers to monomers under these conditions are about 40% and 46% respectively. Second, when protein elipticity as a function of temperature was measured at 222 nm using circular dichroism (data not shown), both forms of GroEL exhibited identical melting curves with transition points at 67°C.

The biochemical activities of the two forms of the protein were then compared. GroEL has been demonstrated to bind to pre-β-lactamase and to facilitate its conversion to mature β-lactamase in the presence of membrane vesicles (Bochkareva *et al.*, 1988). A membrane-translocation assay was not performed using $GroEL_{tr}$ but both forms of the protein are equally able to bind pre-β-lactamase newly synthesized *in vitro* (Fig. 4). GroEL possesses an ATPase activity which is inhibited by GroES (Chandrasekhar *et al.*, 1986; Viitanen *et al.*, 1990); GroES inhibits the ATPase activity of both protein forms equally (data not shown). However, there is a difference between the two forms in the rate at which they hydrolyse ATP (Fig. 5). $GroEL_{tr}$ hydrolyses ATP about 1.5-fold more slowly than does $GroEL_{wt}$. This is the only difference in *in vitro* behaviour manifested by the two forms of the protein which we have been able to identify.

Table 1. Bacterial strains, phages and plasmids

Strain/Phage/ Plasmid	Genotype/Relevant genotype/ Description	Source/ Reference
Strain		
DH1	F⁻, *thi1, relA1, recA1, endA1, spoT1, hsdR17, gyrA96, supE44*	Hanahan (1983)
DL307	F⁻, *recD*	D. Leach
MM22	F⁻, *argG6, asnA31, asnB32, hisG1, leuB6, metB1, pyrE, gal6, lacY1, xyl17, supE44, thuA2, gyrA*	Laboratory stock
MM306	*purA*	Masters (1977)
NL1	PC0698, *purA*⁺	P1 transduction
NL13	NL1 *recA56, srlC300*::Tn*10*	Conjugation[a]
NL30	NL1 *groES30*(Ts)	P1 transduction[b]
NL44	NL1 *groEL44*(Ts)	P1 transduction[b]
NL100	NL1 *groEL100*(Ts)	P1 transduction[b]
NL131	NL1 *groES131*(Ts)	P1 transduction[b]
NL192	NM306 *purA*⁺	This work
NL192Ω	NL192 *delgroESL*::Ω(Spc/Str^R) Requires *groE in trans*	This work
NL193Ω	NL192 ΩTn*7*(Tmp^R) Requires *groE in trans*	This work
NL302	NL30, *recA56, srlC300*::Tn*10*	Conjugation[a]
NL441	NL44, *recA56, srlC300*::Tn*10*	Conjugation[a]
NM306	MM306 *metB*⁺	P1 transduction[b]
PC0698	*purA45*	Coli Genetic Stock Center
Bacteriophage		
λ*sidA*	*lac-5, att*⁺, *imm-21, cI*⁺, *ninR-5* 8.1 kb *groE*⁺ insert	Jenkins *et al.* (1986)
T4	Wild type	Laboratory stocks
T5	Wild type	Laboratory stocks
Plasmid		
pBR322	Amp^R, Tet^R, pMB1 replicon	Bolivar (1978)
pGroESL	2.1 kb *groESL*⁺ *Eco*RI–*Hin*dIII fragment cloned into pACYC	Goloubinoff *et al.* (1989)
pGroE4	pACYC+8.1 kb *groE*⁺ *Eco*RI fragment	Lissin *et al.* (1990)
pGT3270	pJF118EH+2.1 kb *groE*⁺ insert	This work
pGTHC18	pJF118EH+5.0 kb *groES*⁺, *ELtr* insert	This work
pGTIR88	pJF118EH+5.0 kb *groE*⁺ insert	This work
pHC18	pVH1+8.1 kb *groES*⁺, *ELtr* insert	This work
pHC23	As pHC18, but with a 300 bp *Pvu*II deletion (see text)	This work
pHCF3	pML31+8.1 kb *groES*⁺, *ELtr* insert	This work
pHCΩ1	pHC23, *groE* genes replaced with Ω fragment (Spc/Str^R)	This work
pHP45Ω	Amp^R, pBR based plasmid carrying 2.0 kb Ω fragment (Spc/Str^R)	Prentki and Krisch (1984)
pIR88	pVH1+8.1 kb *groE*⁺ insert	I. R. Oliver
pIRF1	pML31+8.1 kb *groE*⁺ insert	This work
pJF118EH	Amp^R, cloning/expression vector *lacI*^{q}, *tac* promoter. EH refers to the orientation of the polylinker	Furste *et al.* (1986)
pJM32	Amp^R, Tet^S, 8.1 kb *Bam*HI deletion derivative of pND5 carrying *groES*⁺ and N'-portion of *groEL* fused to *tet* on a 3.7 kb *Eco*RI, *Bam*HI fragment	March (1988)
pML31	Kan^R, 16 kb mini-F derivative	Lovett and Helinski (1976)
pND5	Amp^R, Tet^R, Chl^S derivative of pBR325 carrying the 8.1 kb *Eco*RI *groE*⁺ insert	Jenkins *et al.* (1986)
pVH1	Kan^R, *lacI*^{q} ColD-based replicon	M. Bagdasarian

a. *recA56* donor: JC10–240 obtained from A. J. Clark.
b. *groE* alleles introduced by P1 transduction from strains obtained from C. Georgopolous (Tilly and Georgopolous, 1982) or R. W. Hendrix.

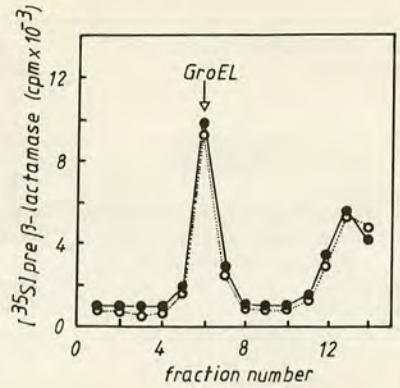

Fig. 4. Post-translational interaction of newly synthesized [^{35}S]-pre-β-lactamase with 1 μM GroEL$_{wt}$ (●) or GroEL$_{tr}$ (O). An S30 extract from which GroEL had been removed by antibody precipitation was used for synthesis. Reaction mixes were analysed by ultra-centrifugation in a sucrose gradient as described in Bochkareva *et al.* (1988).

Construction and behaviour of a strain deleted for the groELS *genes*

pHCF3 and pHC18 were transformed into NL44 and NL100, temperature-sensitive mutants of *groEL*. The transformants, unlike their parents, or siblings transformed with vector plasmids, were able to form colonies and plate lambda at 42°C. Thus *groEL*$_{tr}$ appears to be able to complement *groEL(ts)* mutations, even when contained on a low-copy-number plasmid. The ability of *groEL*$_{tr}$ to complement *groEL(ts)* suggests that the GMR tail motif of the protein has a dispensable function. Since, however, the active form of GroEL is believed to be a 14-mer it is possible that the temperature-sensitive protein and GroEL$_{tr}$ could form a mixed multimer which is active and temperature-stable. Furthermore, recombination between the two mutated genes might occur and yield some wild-type protein. To eliminate these possibilities we constructed a strain from which the chromosomal copy of *groELS* had been deleted in order to study the phenotypes conferred by plasmid-borne *groE* genes. We adapted the method of Kulalauskas *et al.* (1991) to replace the chromosomal *groELS* genes in NM306-(pIRF1) by the Spc^{R}Str^R (spectinomycin-, streptomycin-resistance)-conferring Ω fragment (Prentki and Krisch, 1984). This strain, NL192Ω(pIRF1), survives because complementing *groE* genes are provided on a mini-F plasmid (pIRF1). Southern hybridization was used to confirm that the replacement was successful (Fig. 6). In order to determine whether GroEL$_{tr}$ can replace the wild-type protein, we attempted to transduce the chromosomal deletion into NM306(pHCF3) which expresses GroEL$_{tr}$ from a mini-F plasmid.

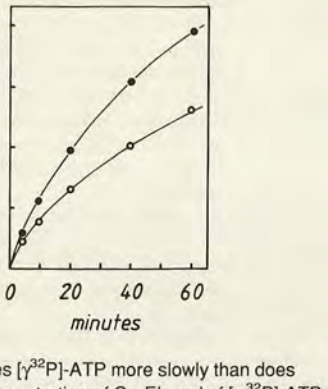

Fig. 5. GroEL$_{tr}$ (O) hydrolyses [γ^{32}P]-ATP more slowly than does GroEL$_{wt}$ (●) at 25°C. The concentration of GroEL and of [γ^{32}P]-ATP are 0.036 μM and 0.1 mM respectively. The ATPase assay was carried out as described in Viitanen *et al.* (1990).

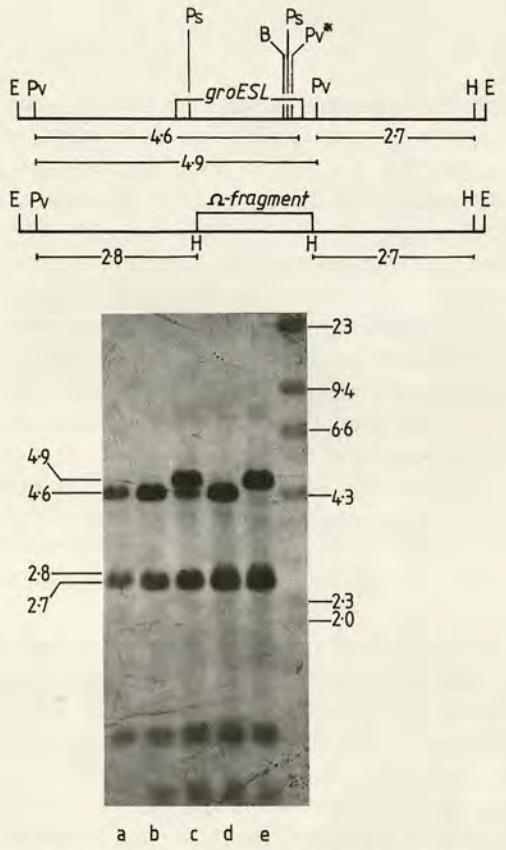

Fig. 6. Verification of the chromosomal *groE* deletion in NL192Ω.
Top: 8.1 kb *Eco*RI fragment showing the location of *groE*. The boxed region is the sequence reported in Hemmingsen *et al.* (1988). Restriction sites shown are those relevant to the constructions described as well as to the deletion verification. E, *Eco*RI; Ps, *Pst*I; B, *Bam*HI; H, *Hin*dIII; Pv, *Pvu*II; Pv*, *Pvu*II site lost when termination sequences were inserted. Sizes are in kb.
Centre: Expected structure after Ω replacement of *groE* in pHC23.
Bottom: Southern hybridization using the entire *Eco*RI (*groE*$^+$) fragment prepared from pND5 (mixed with λ DNA) as probe. Chromsomal DNAs were cut with *Eco*RI, *Hin*dIII and *Pvu*II. Lanes: a, NL192; b, NL192(pIRF1); c, NL192(pHCF3); d, NL192Ω(pIRF1); e, NL192Ω(pHCF3). Molecular weight markers are *Hin*dIII cut λ DNA.

Table 2. P1 transduction of the *groELS* gene replacement.

	No. PurA$^+$ progeny		No. Spc^R progeny		Linkage (%)	
Recipient strain	A	B	A	B	A	B
MM306(pML31)	74	238	0	0	0	0
MM306(pIRF1)	81	249	10	37	12	15
MM306(pHCF3)	92	282	12	45	13	16

P1 lysates were prepared on NL192Ω(pIRF1) and PurA$^+$ transductants selected and scored for Spc^R. A and B are separate experiments; in experiment B recipients were derived from NM306.

A P1 transducing lysate was prepared on NL192Ω (pIRF1) and used to transduce NM306(pHCF3). Spc^{R}Pur$^+$ progeny were selected in order to obtain chromosomal *groE* gene replacements (*purA* is linked to *groE* in transductional crosses and is not within the plasmid insert). As can be seen in Table 2 the linkage of the two markers was not reduced in the pHCF3 relative to the pIRF1 recipient, indicating that both strains can tolerate deletion of the chromosomal *groE* genes and showing that *groEL*$_{tr}$ can substitute for the *groEL*$^+$ gene. Progeny of this cross were tested by Southern hybridization to further confirm that they contain *groEL*$_{tr}$ as their only *groEL* gene (Fig. 6).

Phenotypes of strains with GroEL$_{tr}$ *as their only form of GroEL*

The fact that normal numbers of normally sized colonies of *groEL*$^+$ deleted, *groEL*$_{tr}$ progeny were obtained in the transductional cross described above proves that the GMR tail of GroEL is not essential for growth under the selective conditions used (glucose-casamino acid plates at 30°C). We found in addition that the ability of NL192Ω(pHCF3) to grow on minimal agar supplemented with one of a variety of carbon sources (glycerol, D-mannitol, proline+alanine, succinate, glucose, maltose or galactose) or on L-broth is unaffected at either 30°C or 42°C when compared with NL192Ω(pIRF1).

A variety of phenotypic defects have been associated with mutations in the *groE* genes and we therefore attempted to determine whether truncation of GroEL results in related defects. GroEL is required for the successful assembly of phages T4, T5 and λ and *groE(ts)* mutants fail to support phage growth, even at temperatures permissive for cell growth (Georgopoulos *et al.*, 1973). The plating efficiencies of these phages are not reduced in NL192Ω(pHCF3) at either 30°C or 42°C, demonstrating that GroEL$_{tr}$ can support normal phage duplication. GroEL has been implicated in the transport of β-lactamase from cytoplasm to periplasm (Bochkareva *et al.*, 1988; Kusukawa *et al.*, 1989). Although we have already shown that GroEL$_{tr}$ can bind to pre-β-lactamase *in vitro*, we also tested whether β-lactamase is found within the periplasmic compartment in *groEL*$_{tr}$ strains *in vivo*. The periplasmic fractions of strains were prepared as described and β-lactamase assayed in these fractions and in sonicated whole cells. As a control for the efficiency of the fractionation procedure parallel *groE*$^+$ cultures were fractionated and assayed for basal and induced β-galactosidase levels: 13% of basal and 25% of induced β-galactosidase activities were recovered in the periplasmic fractions defining these as the maximum levels of cytoplasmic contamination in these fractions. In contrast, the periplasmic fractions contained essentially all the β-lactamase activity and no differences were found between the strains.

The *groEL* gene belongs to the heat-shock regulon of *E. coli* (Yamamori and Yura, 1980; Neidhardt *et al.*, 1983) and is induced in response to shifts to higher temperatures (Neidhardt *et al.*, 1981). We therefore tested whether the ability of *E. coli* to survive exposure to high temperature requires the intact form of GroEL. Table 3 shows that although resistance to high temperature is reduced by deletion of the chromosomal gene (this could be due to effects on the replication of the F-plasmid which carries the *groE* genes) the *groEL*$^+$ deletion strain survives no better than does the isogenic *groEL*$_{tr}$ strain. Similar results were found when the strains were pretreated at 42°C for 30 min prior to incubation at 56°C (data not shown). Although this would not be surprising in view of the report that σ^E rather than σ^{32}-regulated genes are primarily responsible for thermotolerance (Erickson and Gross, 1989) σ^{32}-deleted strains have also been reported to exhibit greatly reduced thermotolerance (Jenkins *et al.*, 1991).

As a final test for a phenotypic property attributable to the *C*-terminal GroEL sequence we decided to look more closely at the growth of NL192Ω(pIRF1) and NL192Ω (pHCF3). Both of these strains grow at the same rates at 30°C and 42°C as measured by optical density increase during exponential growth. Small differences in growth rate, however, might not be detected by this relatively

Table 3. Survival at 56°C of strains lacking a chromosomal *groEL* gene.

	Plating Efficiency After Incubation at 56°C for:				
Strain	0 min	5 min	10 min	15 min	30 min
NL192(pML31)	1.0	2×10^{-1}	3×10^{-2}	5×10^{-3}	6×10^{-4}
NL192Ω(pIRF1)	1.0	6×10^{-2}	3×10^{-3}	1×10^{-3}	1×10^{-4}
NL192Ω(pHCF3)	1.0	4×10^{-2}	2×10^{-3}	1.5×10^{-3}	1×10^{-4}

Cultures were grown in broth to mid-log phase at 30°C before transfer to 56°C. Aliquots were removed at the indicated times and serial dilutions of these were plated out at 30°C. Values given refer to the fraction of viable cells remaining with respect to the initial number of cells treated.

Table 4. Co-culture of *groEL*$^+$ and *groEL*$_{tr}$ strains.

		0 h			24 h			48 h			72 h		
	Temp.	total	Tmp^R	ratio	total	Tmp^R	ratio	total	Tmp^R	ratio	total	Tmp^R	ratio
A	30°C	130	63	1.1	172	82	1.1	111	39	1.9	160	56	1.9
	42°C				100	12	7.3	138	18	6.7	139	3	45
B	30°C	50	23	0.9	128	76	1.5	134	90	2.1	132	91	2.2
	42°C				100	84	5.3	135	121	8.7	146	145	145

30°C cultures were diluted at 10^4 cells ml^{-1} into LB; equal volumes of pairs were mixed and incubated for 24 h. The mixed cultures were sampled and diluted to 10^4 cells ml^{-1} daily. Colonies from non-selective plates were patched on to trimethoprim. A, NL192Ω(pIRF1)+NL193Ω(pHCF3); B, NL193Ω (pIRF1)+NL192Ω(pHCF3). Ratios are of *groEL*$^+$/*groEL*$_{tr}$. The experiment was repeated several times with similar results.

crude measure. To test, therefore, the ability of GroEL$_{tr}$ to fully substitute for GroEL$_{wt}$ we decided to co-culture the two strains to determine whether NL192Ω(pHCF3) can compete successfully with NL192Ω(pIRF1) when the two are co-cultured in broth.

In order to do this a host strain distinguishable from, but otherwise isogenic with, NL192Ω was required. NL193Ω was constructed by transducing NL192Ω to trimethoprim (Tmp)-resistance using a Tn*7*-containing strain as donor. NL192Ω(pIRF1) and NL193Ω(pHCF3) and, separately, the reciprocal pair, were co-cultured as described and the proportions of each determined by measuring the ratio of Tmp^R:Tmp^S cells (Table 4). The results were striking. When the pairs were co-cultured at 42°C the strain producing GroEL$_{wt}$ quickly came to predominate in the mixture. In this experiment periods of exponential growth alternated with periods in stationary phase. The difference between the strains could thus have been in growth rate during exponential growth, because of optical density achieved during stationary phase, or because of ability to recover from stationary phase. Measurements were performed on each of the strains separately and were unambiguous; the difference between the strains lies in the last of these processes. Strains expressing GroEL$_{tr}$ reach the same final OD_{540} and contain the small cells characteristic of stationary phase (Lange and Hengge-Aronis, 1991) but require two to four generation times longer to recover from stationary phase when grown to stationary phase at 42°C than do the *groEL*$^+$ strains (Fig. 7). This is because of events that occur during stationary phase at 42°C since 42°C cultures inoculated from 30°C parental cultures showed no growth lag and cultures inoculated from the 42°C stationary cultures for growth at 30°C behave as do those reinoculated for further growth at 42°C (data not shown).

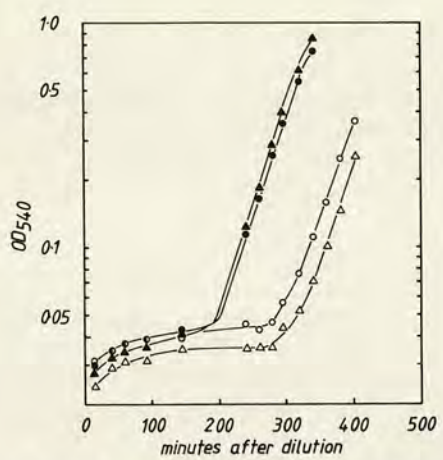

Fig. 7. Strains reliant on GroEL$_{tr}$ are delayed in recovery from stationary phase. LB cultures were inoculated with 10^4 cells ml^{-1}, incubated with shaking at 42°C for 46 h, diluted 100-fold into fresh LB and the OD_{540} followed. ▲ NL193Ω(pIRF1), ●, NL192Ω(pIRF1); ○, NL193Ω(pHCF3), △ NL192Ω(pHCF3).

GroEL$_{tr}$ *does not suppress dnaA(ts) mutations*

Overexpression of the *groE* genes can suppress many *dnaA(ts)* mutants permitting growth at high temperature (Jenkins *et al.*, 1986; Fayet *et al.*, 1986). In order to test whether GroEL$_{tr}$ can also mediate such suppression MM22, a *dnaA46 recA* strain was transformed with the high-copy-number plasmids pGTIR88 and pGTHC18 containing *groEL* and *groEL*$_{tr}$ respectively. Progeny were selected for drug resistance at 30°C and tested for growth at 40°C or selected directly at 40°C. Results were clear: transformants were obtained with all plasmids at 30°C but only with those containing *groEL*$^+$ at 40°C (Table 5). Of the transformants obtained at 30°C only those with the *groEL*$^+$ gene grew at 40°C on restreaking. It is also worth noting that the progeny that had received *groEL*$_{tr}$ grew well at 30°C while those that had received the wild-type, suppressing gene exhibited cold-sensitivity as observed previously (Jenkins *et al.*, 1986). This cold-sensitivity has been attributed to over-initiation of replication at 30°C in

Table 5. *groEL*$_{tr}$ can complement *groEL*$_{ts}$ but cannot suppress *dnaA46*.

Recipient strain	Plasmid	No. of Colonies 30°C	No. of Colonies 42°C	Transformants μg^{-1} DNA 30°C	Transformants μg^{-1} DNA 42°C
NL441	pJF118	1500	0	1.5×10^4	0
(*groEL44*	pGTIR88	2700	2500	2.7×10^4	2.5×10^4
recA56)	pGTHC18	2400	2400	2.4×10^4	2.4×10^4
		30°C	40°C	30°C	40°C
MM22	pJF118	2100	0	2.1×10^4	0
(*dnaA46*	pGTIR88	83[a]	1800	8.3×10^2	1.8×10^4
recA56)	pGTHC18	1900	0	1.9×10^4	0

Transformation mixtures each contained 100 ng of DNA. Reduced nos of colonies at (a) is because of the cold-sensitivity of this combination.

strains in which suppressible *dnaA*(*ts*) mutations are combined with suppressing levels of GroELS (Katayama and Nagata, 1991). Our results here confirm that the cold-sensitive phenotype is displayed only when suppression is possible and shows that suppression requires the GMR tail of the GroEL protein.

Discussion

GroEL, almost all of its homologues, and many members of the HSP70 class of heat-shock proteins all share a striking *C*-terminal motif, namely the GMR tail. The evolutionary conservation of this tail suggests that it is likely to have a functional role. We have removed the tail from the *E. coli* GroEL protein to create a truncated derivative, GroEL$_{tr}$. We show here that GroEL$_{tr}$ is functionally able to replace GroEL. Thus, the *C*-terminal tail does not appear to be needed for normal exponential growth at 30°C or 42°C in complex or defined media; nor for assembly of GroEL 14-mers *in vivo* or their stability *in vitro*. It is not required for translocation of β-lactamase across the cell membrane, for GroEL ATPase activity or GroEL interaction with GroES. It is not required for propagation of GroEL-dependent bacteriophages. Thus, if the GMR tail has a functional role it is likely to be a relatively subtle one.

We have observed two clear *in vitro* defects associated with the truncated protein. These are that high levels of this form of GroEL cannot restore the activity of temperature-sensitive forms of the DnaA protein and that cells producing only the truncated form of the protein show a marked delay in the transition from the stationary to the exponential growth phase. Restoration of DnaA activity requires overproduction of GroES and GroEL, proteins which are already extremely abundant; it might thus be argued that GroEL$_{tr}$ is ineffective because it is less abundant, perhaps because it has reduced stability. So far as we can tell this is not the case (see Fig. 2). Both forms of the protein are heavily over-produced to similar extents; both forms of the protein are to be found in the characteristic 14-mers; both types of 14-mer are equally stable (at least *in vitro*). GroEL ATPase activity is required for the release of bound proteins from GroEL in their folded, functionally active states (for a review see Hartl *et al.*, 1992). It is thus possible that suppression of *dnaA(ts)* requires very high levels of this activity and that the inability of GroEL$_{tr}$ to suppress *dnaA(ts)* is due to insufficient ATPase activity. On the other hand it is possible that the GMR tail has a specific role in *dnaA(ts)* suppression.

Recent work on the biochemical changes that accompany the onset of stationary phase in *E. coli* demonstrate that stationary phase is a bona fide developmental state characterized by the synthesis of a specific spectrum of proteins (Groat *et al.*, 1986). Amongst the proteins whose concentrations are markedly increased during stationary phase are the σ^{32}-dependent heat-shock proteins which include GroEL (Jenkins *et al.*, 1991). As Siegele and Kolter (1992) point out, the starved cell must synthesize any proteins required to maintain viability during starvation and must also make those required for recovery and resumption of growth when nutrients again become available. GroEL and its *C*-terminal tail appear to have a role in these processes.

What sort of function could the *C*-terminal tail have? Biochemically, a series of glycines and methionines is likely to be both hydrophobic and flexible. The hydrophobic nature of the tail suggests that it is not free in the cytoplasm but shielded by interaction with a hydrophobic target. Rippmann *et al.* (1991) suggest that in HSP70s this motif might react with the HSP70 peptide binding site, leading to HSP70 oligomer formation. We have found, in data-base searches, that similar glycine-rich quasi-repeats (with or without methionine) characterize other protein groups, most notably the keratins. Steinert *et al.* (1991) suggest that, in these proteins, the glycine-rich domains are organized into loops in which the hydrophobic residues (i.e. methionines) stack and the glycine repeats loop between them to create a flexible structure akin to a molecular spring. They hypothesized further that individual molecules may interact by way of these glycine-loop motifs to form arrays.

So what might be the role of the *C*-terminal GMR motif in GroEL? Fourteen monomers of GroEL must associate to form the characteristic 14-mer. Does the *C*-terminal tail assist in this association? If oligomerization were reduced this could explain why *dnaA* mutants fail to be suppressed by GroEL$_{tr}$. Although we find GroE$_{tr}$ assembled into oligomers, we have not quantitatively determined whether all the protein is assembled.

An alternative possibility is that the tail is required to associate with certain substrates or with co-acting chaperones. Is it possible that DnaA, because of its structure

or location (DnaA has been reported to be at least partly membrane located (Sekimizu *et al.*, 1988)), interacts first with the tail of GroEL and only secondarily with the rest of the molecule? Or perhaps the tail of GroEL promotes an interaction with, for instance, DnaK (which does not itself possess the *gly.met* repeats characteristic of some of its eukaryotic HSP70 relatives (Bardwell and Craig, 1984)) and an association of these two chaperone proteins is required for *dnaA* suppression.

The dispensability of the GroEL GMR does not extend to adjacent sequences. Removal of a further 14 *N*-terminal amino acids (N. F. McLennan and M. Masters, unpublished) results in a protein unable to complement *groEL(ts)* mutations. Thus GroEL does not possess an extensive inessential *N*-terminal region.

In conclusion, we find that the conserved *C*-terminal sequence of GroEL is not required for normal growth and could thus be considered expendable. However, on closer examination this sequence can be shown to confer a selective advantage, an advantage presumably sufficient to result in its conservation.

Experimental procedures

Media

Luria Broth (described in Masters, 1970) was used for liquid culture growth and solidified with 1.5% agar in plates. Ampicillin (50 μg ml^{-1}), kanamycin (30 μg ml^{-1}), spectinomycin (50 μg ml^{-1}), and streptomycin (20 μg ml^{-1}) were added to selective media as required. Minimal medium (VB) was that of Vogel and Bonner (1956). Trimethoprim was added to minimal plates at 50 μg ml^{-1}. For manipulations involving λ phage, L-broth was supplemented with 20 mM $MgSO_4$ and 0.2% maltose. For phage P1, L-broth was supplemented with 2 mM $CaCl_2$.

Genetic and biochemical methods

P1 lysate preparation and transduction were carried out as previously described (Masters, 1970). Phage λ was prepared by plate lysates or when larger quantities were required, by ultraviolet induction as described by Wilson *et al.* (1977). Small-scale preparation of plasmids for screening and checking strains was by the method of Birnboim and Doly (1979). Bulk preparation on CsCl-ethidium bromide gradients, restriction of DNA, agarose gel electrophoresis and SDS–PAGE were as described by Maniatis *et al.* (1982). Transformation of plasmid DNA into *E. coli* was performed using the method of Chung *et al.* (1989). This method was found to give better transformation efficiencies with temperature-sensitive strains than conventional $CaCl_2$ methods possibly because heat-shock is not required. Enzymes and biochemicals were purchased from Boehringer Mannheim, New England Biolabs or Sigma unless stated otherwise.

Plasmid construction

pIR88 was made by ligating the 8.1 kb *Eco*RI *groE*$^+$ fragment from pND5 into the unique *Eco*RI site in pVH1 (ColD, kanamycin-resistant (Kan^R)). The resulting 13.1 kb plasmid complements *groES* and *groEL* temperature-sensitive mutants both for high temperature and phage λ growth.

pHC18 was made by partially digesting pIR88 with *Pvu*II and purifying the linearized plasmid band, cut from an agarose gel, with GeneClean (Bio101). This DNA was blunt-end-ligated to a 20-fold molar excess of the Universal Translation Terminator oligonucleotide (5'-GCTTAATTAATTAAGC-3' purchased from Pharmacia (prod. no. 27–4890)). The ligation mix was transformed into DH1 and the construction verified by DNA restriction analysis and SDS–PAGE analysis of the protein product. pHC23 was also isolated. This plasmid resembles pHC18 but lacks the downstream 300 bp *Pvu*II fragment which encodes the *C*-terminal portion of GroEL. Strains carrying pHC18 or pHC23 are phenotypically identical.

pIRF1 and pHCF3 were made by partially digesting pML31 (mini-F, Kan^R) with *Eco*RI, gel-purifying the linearized plasmid as above and ligating this with the 8.1 kb *Eco*RI *groE*-containing fragments from pIR88 and pHC18 respectively. The ligation mix was transformed into NL302 (*groESts*) at 42°C to select plasmids that carry *groE*$^+$ genes.

pGT3270 was made by ligating the 2.1 kb *Eco*RI–*Hin*dIII *groE* fragment from pGroESL to the *Eco*RI–*Hin*dIII sites of pJF118EH (ColE1, ampicillin-resistant (Amp^R)).

pGTIR88 and pGTHC18 were made by ligating the 3.0 kb *Bam*HI–*Hin*dIII fragment, which carries the distal (3') end of the *groEL* gene, from pIR88, and pHC18, respectively, into the *Bam*HI–*Hin*dIII sites of pGT3270. Ligation mixes were transformed into DH1 and constructions were verified by DNA restriction analysis and by SDS–PAGE of their protein products.

pHCΩ1 was constructed by restricting pHC23 with *Pst*I and gel purifying the 11 kb fragment lacking the *groE* genes (see Fig. 6). This DNA was mixed with pHP45Ω which had been restricted with *Bam*HI, and with a 20-fold molar excess of a *Pst*I–*Bam*HI adaptor oligonucleotide purchased from New England Biolabs. The ligation mix was transformed into DH1 selecting for Kan^R, Spc^R and Str^R. Absence of *groE* was genetically verified by failure to complement the *groE* mutations in NL441 (*groELts*) and NL302 (*groESts*).

Southern blotting

Probe DNA was labelled using bio-11-dUTP (Sigma); the Boehringer Mannheim Nick Translation Kit was used according to the manufacturer's instructions. Five micrograms of *E. coli* chromosomal DNA, restricted with the appropriate enzymes, was used per gel lane. Transfer of the DNA from agarose to nitrocellulose was by the ammonium acetate method of Smith and Summers (1980). Hybridization and detection of the biotinylated probe DNA was as recommended in the BRL blu-GENE Biotinylated Probe Detection Kit.

Construction of a groE *deletion strain*

A *groE* deletion strain was constructed using an adaption of the method of Kulakauskas *et al.* (1991). The strain DL307 was lysogenized with λsidA (*groE*$^+$) and transformed with pHCΩ1. The phage was induced with ultraviolet and the resulting lysate

used to lysogenize DL307 selecting for Spc/Str-resistance. Spc/Str-resistant phage would arise by homologous replacement of the *groE* genes on λ*sidA* by the Ω fragment. Spc/Str^R lysogens were induced with ultraviolet and the resultant phages tested for their ability to form plaques on *groEts* (λ^R) strains. All the analysed clones were carrying a *groE*$^-$ Spc/Str^R λ phage. One of these phages (λ*sid*Ω1) was used to infect DL307 lysogenized with λ*sidA* and Spc/Str-resistant recombinants were selected in liquid culture. Expected amongst the resistant progeny would be clones in which the chromosomal *groE* genes have been replaced by Ω-containing DNA. An aliquot of this mixed culture was used to make a P1 lysate which was then used to transduce MM306 (*purA*$^-$) pIRF1 to *purA*$^+$ and Spc/Str resistance. *purA* and *groE* are 15% cotransducible. Five P1 lysates were made on the progeny obtained and again used to cotransduce *purA*$^+$ and Spc/Str resistance. Two of the lysates produced cotransductants only of GroE diploid recipient strains as predicted for a donor with the desired Ω replacement of *groE*$^+$. The correct construction was verified by Southern analysis of the chromosomal DNA.

Release of periplasmic β*-lactamase by osmotic shock*

In order to determine the amount of β-lactamase secreted into the periplasm of *E. coli* the method of Neu and Heppel (1965) was adopted in which osmotic shock is used to liberate periplasmic proteins. Twenty-five millilitres of L-broth was inoculated with the appropriate strains of bacteria and grown with vigorous agitation until an OD_{540} of 0.5 was achieved. The cells were harvested by low-speed centrifugation at 4°C, and the pellet resuspended in 10 ml of 50 mM NaCl, 70 mM phosphate buffer (pH 7.0) and recentrifuged. The pellet was resuspended in 5 ml of 20% sucrose, 0.03 M Tris-HCl (pH 8.0) and 1 mM EDTA. The suspension was incubated in a 100 ml flask on a rotary shaker at 0°C for 10 min and the cells harvested as above. The thoroughly drained cell pellet was resuspended in 5 ml of distilled water, shaken at 0°C for 10 min and centrifuged. The resulting supernatant (termed cold water wash) was assayed for β-lactamase activity.

Determination of β*-lactamase activity*

In order to quantify β-lactamase present in the cellular periplasmic fraction a colorimetric assay system employing 87/312 (purchased from Oxoid) was used (O'Callaghan *et al.*, 1972). The osmotic shock cold water washes were diluted in 0.01 M phosphate buffer (1 μl per ml) and 87/312 added to a concentration of 6 μl ml^{-1} in a final reaction volume of 3 ml. The rate of appearance of the red product was measured at 500 nm at 25°C.

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